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The Journal of Biochemistry, Vol. 46, No. 12, 1959

TRYPSINOGEN-KINASE IN ASPERGILLUS ORYZAE

V. ON THE MECHANISM OF ACTIVATION

By KAZUO NAKANISHI

(From Takamine Laboratory, Sankyo Co., Ltd., Tokyo)

(Received for publication, February 4, 1959)

It was previously reported that aspartic acid was liberated in trypsinogen activation by the trypsinogen-kinase found in *Aspergillus oryzae.* In a later investigation on the purification of this enzyme, this trypsinogen-kinase was identified as an acid-protease. Various proteolytic properties of this enzyme have also been investigated (*1*). The mechanism of the trypsinogen activation is of considerable interest from the standpoints of the chemical structure of proteins and of the enzyme chemistry. Desnuelle (*2*) postulated that a Val-peptide was cleaved off from the N-terminal of trypsinogen in its activation. Neurath was able to demonstrate that in the activation of trypsinogen by trypsin (*3*) or in the autoactivation of trypsinogen (*4*), Val-$(Asp)_4$-Lys was cleaved off from the N-terminal of trypsinogen. Yamashina (*5*) found a similar cleavage of a Val-peptide in the activation by enterokinase. Both the kinase and trypsinogen used in the author's previous investigations on the mechanism of activation were crude preparations. In the present study, this activating mechanism was reinvestigated with purified materials and was compared with that by trypsin or enterokinase.

EXPERIMENTALS

Trypsinogen—G.B.I. trypsinogen crystals, sufficiently dialysed against $2\times10^{-3}\,N$ HCl, followed by lyophilization. Trypsin activity of this preparation was practically none. As the *N*-terminal, no amino-acid other than valine could be detected by the DNP method. When activated, it exhibited the same activity as that of trypsin crystals.

Trypsinogen-kinase—Purified samples obtained by the ion exchange resin method as previously described. (*1*).

Determination of Total Amino-acid—Stein-Moore method (*6*).

Determination of Respective Amino-acid—Microbioassay method.

Determination of Trypsin—The same method as previously reported.

DNP Method—DNP-protein was obtained by Sanger's method (*7*), hydrolysed, extracted with ether, and the ether solution was analysed for DNP-amino-acid by paper chromatography method of Blackburn (*8*).

Experiment I. Determination of Trypsinogen Activation and of Total Ninhydrin-Positive Products

Trypsinogen was mixed with trypsinogen-kinase while being cooled with

ice water, and was activated at 0°. At suitable intervals the amount of ninhydrin-positive products in the 5 per cent TCA soluble fraction was determined. At the same time, the amount of activated trypsin was measured. The results are shown in Fig. 1. In both cases, activated trypsin and total ninhydrin-positive products showed a parallel increase, without any of the complicated reactions, *e.g.* secondary decomposition.

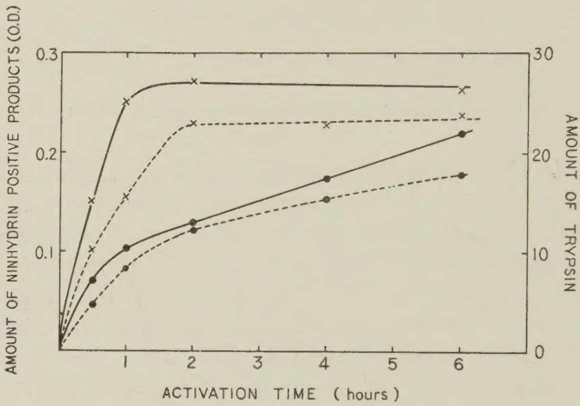

FIG. 1. Determination of the trypsinogen activation and of the total ninhydrin positive products.

Trypsinogen, 42 mg., was dissolved in 6 ml. of Walpole buffer solution, pH 3.5, and was mixed with 6 ml. of trypsinogen-kinase aqueous solution while being cooled with ice water, and activation was carried out at 0°. At suitable intervals, 1 ml. of this solution was taken, mixed with 2 ml. of 7.5 per cent TCA and filtered after standing at 35° for 30 minutes. The amount of ninhydrin-positive products in the filtrate was determined. At the same time, the amount of activated trypsin was measured. (•): kinase 5 μg./ml., ($\times$): kinase 15 μg./ml., (——): ninhydrin-positive product, (- - - -): trypsin.

Experiment II. Paper Chromatography of the Ninhydrin-Positive Products

Activation was carried out under the same condition as shown in Experiment I. At suitable intervals Amberlite IR-120 (H-form) was added. The pH of the solution decreased to 1.4. In preliminary experiments it was found that under these conditions all amino-acids were completely adsorbed and the activation of trypsinogen-kinase ceased to occur. The resin was then adequately washed with water and extracted twice with NH_3 aq. The extracted solution was evaporated to dryness under reduced pressure. One-dimensional paper chromatography was then applied to this sample. As shown in Fig. 2, no spots appeared at the zero hour. At the points of 19, 63 and 91 per cent activation three similar spots corresponding to valine, aspartic acid and lysine were observed, of which the spot corresponding to aspartic acid appeared to be the largest. With progress of activation, the relative color intensities of these three spots showed no observable change,

and the color intensity in general became stronger as a whole.

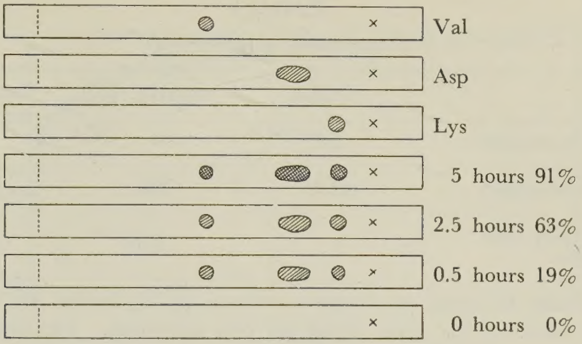

FIG. 2. Paper chromatography of the ninhydrin-positive products. 70 mg. each of trypsinogen was dissolved in 20 ml. of 5-times diluted Walpole buffer solution, pH 3.5, and 50 μg. of kinase was added to each solution and activation was carried out at 0°. After 0, 0.5, 2.5 and 5 hours 4 g. of Amberlite IR-120 (H-form) was added and the mixture was shaken at room temperature for 1 hour. The resin was then washed with water and extracted twice with 15 ml. of 5 N NH_4OH. The extracted solution was evaporated to dryness under reduced pressure. One-dimensional paper chromatography was applied with buthyl alcohol; acetic acid : water (4 : 2 : 1).

Experiment III. Determination of Amino-acids

In Experiment II, three spots were observed in the chromatograms corresponding to valine, aspartic acid and lysine. The amounts of these three amino-acids were determined. Under the same conditions as shown in Experiment II, the evaporated residue was subjected to analysis by the microbioassay method before and after hydrolysis. The results were shown in Table I. When converted into molal ratio, Val : Lys : Asp becomes 1 : 1.28 : 3.53.

Experiment IV. The N-Terminal Amino-acid of Trypsin Obtainea by Trypsinogen-kinase Activation

Following complete activation by the method described in Experiment II, smallmolecular weight substances were removed by adequate dialysis against 2×10^{-3} N HCl. DNP-trypsin was obtained by the established methods, and was hydrolysed. DNP-amino-acids were extracted with ether, and the ether solution was analysed for amino-acids by paper chromatography. Spots corresponding to leucine and to a minute amount of valine were observed.

Leucine and isoleucine could not be differentiated by this method.

TABLE I

Determination of Amino-acids

	Valine	Aspartic acid	Lysine HCl
Before hydrolysis	5.2 (μg.)	0 (μg.)	6.6 (μg.)
After hydrolysis*	19.4	78.0	38.7
Molar ratio after hydrolysis	1	3.53	1.28

Under the same conditions as shown in Experiment II, samples were taken at the point of 60 per cent activation. The evaporated residue was dissolved in 20 ml. of water and subjected to analysis by the microbioassay method before and after hydrolysis.

* with 3 *N* HCl for 8 hours at 120°.

Experiment V. Comparison of Trypsins Activated by Trypsin and by Trypsinogen-kinase

Complete activation of trypsinogen by trypsinogen-kinase and by trypsin was performed at pH 3.5 and 8.0 respectively and activated trypsins were lyophilized. Then, casein was hydrolysed by both of the trypsins. As shown in Fig. 3, no difference could be seen in the two hydrolysis curves and in the cross experiments. Trypsin obtained through the action of trypsinogen-kinase also readily hydrolysed benzoyl-Arg-NH_2, the specific substrate of trypsin. (Table II)

TABLE II

Hydrolysis of Benzoyl-Arg-NH_2 with Both Trypsins Activated by Trypsin and Trypsinogen-kinase

	Hydrolysis (%)
Trypsin activated by trypsin	84
Trypsin activated by trypsinogen-kinase	86

Benzoyl-Arg-NH_2	3×10^{-2} *M*	1 ml.
pH 8.0 buffer		1 ml.
Trypsin	200 μg./ml.	1 ml.

After hydrolysis at 30° for 24 hours NH_3 was measured by Conway's micro-diffusion method (*9*).

DISCUSSION

Neurath observed that many amino-acids were liberated in the process

of the trypsinogen activation but among them only the amount of Val-$(Asp)_4$-Lys showed an increase in parallel with the progress of activation. He is in

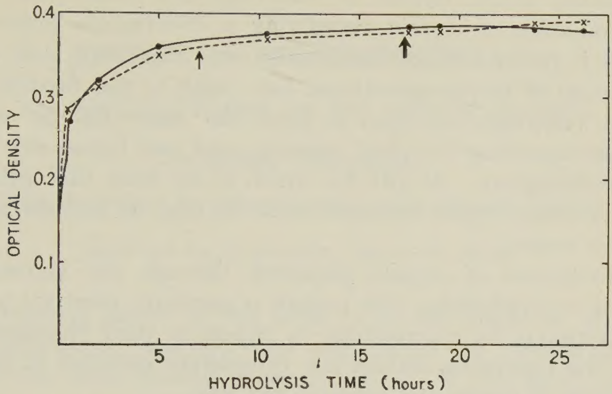

FIG. 3. Hydrolysis courves of both trypsins activated by trypsin and by trypsinogen-kinase.

To 20 ml. of 2 per cent casein $M/10$ Na_2HPO_4 solution, 40 ml of water was added and hydrolysis was carried out at 35° by 1.3 mg. of trypsin which corresponded to 1 mg. of trypsin crystals. After 7 hours, (↑), 1 mg. each of trypsin was added to each 35 ml. of the reactive solution and hydrolysis was allowed to continue for 10 more hrs. to ensure complete hydrolysis. Next (**↑**), to each 20 ml. of the reactive solution, 500 μg. of trypsins were interchanged and added. At suitable intervals, samples were tested by Stein and Moor method.

—— by trypsin, ---- by trypsinogen-kinase.

the assumption that the liberation of other amino-acids was the result of secondary autolysis of trypsin. In the present study, ninhydrin-positive products increased proportionally to the progress of activation and no other spots in the paper chromatograms of the produced amino-acids were observed except for three spots corresponding to valine, aspartic acid and lysine. With the progress of activation, the relative color intensities of these three spots showed no observable change, and the color intensity in general became stronger as a whole. These observations suggest that these three spots are not the result of irregular hydrolysis. Analysis for valine, aspartic acid and lysine in 60 per cent activated solutions showed that valine and lysine in amounts of 5.2 μg. and 6.6 μg. respectively were formed while absolutely no free aspartic acid was observed before hydrolysis with HCl. After hydrolysis the amounts of valine, lysine and aspartic acid increased to as much as 19.4 μg., 38.7 μg. and 78.0 μg. respectively. From these results, it is assumed that, of the amino-acids produced in the activating process, valine and lysine exist as peptides for the most part and partly in the free state, and aspartic acid is present in a combined form but not in free form. The amounts of valine, lysine and aspartic acid after hydrolysis showed a molar ratio of 1 : 1.28 : 3.53. This value approximates to the ratio, 1 : 1 : 4, of peptides obtained by Neurath.

From the results obtained in this study, the mechanism of trypsinogen activation may be presumed as follows. When trypsinogen is activated by trypsinogen-kinase, initial release of Val-$(Asp)_4$-Lys takes place rapidly, following the production of trypsin containing a N-terminal isoleucine. Next, Val-$(Asp)_4$-Lys is partly and gradually split into Val, $(Asp)_4$ and Lys through the specific action of trypsinogen-kinase but $(Asp)_4$ is not further hydrolysed. As $(Asp)_4$ and Val-$(Asp)_4$-Lys seem to have the same Rf. as aspartic acid, three spots, corresponding to valine, aspartic acid and lysine, are obtained on the paper chromatogram. At pH 3.5 which is far from the optimum pH of trypsin, the activated trypsin becomes stable so that no secondary decomposition by trypsin occurs.

As the N-terminal of trypsin produced through the activating process mentioned above is isoleucine, this trypsin is perfectly identical to the trypsin activated by trypsin or by enterokinase in respect to their chemical structures. Furthermore, the enzymatic actions are completely identical as demonstrated by the hydrolysis of casein and benzoyl-Arg-NH_2.

SUMMARY

1. In the activating process of trypsinogen by trypsinogen-kinase, the compound Val-$(Asp)_4$-Lys is initially produced.

2. Val-$(Asp)_4$-Lys is further hydrolysed partly into valine, $(Asp)_4$ and lysine.

3. The activated trypsin is identical with the ordinary trypsin in respect to its enzymatic activity.

4. The previous statement that aspartic acid is liberated in the activation, is now revised herein to the conclusions given above.

The author wishes to express his thanks to Prof. Akabori of Osaka University for his kind guidance.

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The Journal of Biochemistry, Vol. 46, No. 12, 1959

STUDIES ON HOMOGENTISICASE

III. KINETIC STUDIES ON THE ENZYME ACTION

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(Received for publication, March 10, 1959)

In the proceding papers of this series (*1*, *2*) the behavior of ferrous ion in the homogentisicase reaction and the effects of various agents on the enzymatic activity were studied in some detail. It was found, among other things, that the iron bound to the enzyme protein participated in the reaction in the ferrous state, without undergoing a valency change during its participation. It was further inferred that the enzyme molecule possessed at least two types of reactive groups which were indispensable for its activity; namely a sulfhydryl group which has also been recognized by other authors (*3*, *4*) and a phenolic hydroxyl group which is susceptible to the action of tyrosinase.

The investigation to be reported in the present paper was undertaken to obtain further information concerning the mechanism of the homogentisicase reaction by investigating the dependence of the reaction rate upon the concentration of the substrate, ferrous ion and hydrogen ion. The nature of the active groups responsible for the association of the substrate and ferrous ion with the enzyme protein will be discussed based on the analysis of the kinetic data obtained.

METHODS

Homogentisicase was partially purified from beef liver as previously described (*1*) and the Fe^{++}-free enzyme preparation was employed throughout the present investigation. The reaction rate was determined from the tangent at zero time of the oxygen uptake-time curve and was expressed in terms of μl. oxygen consumed per minute per mg. protein. The manometric readings were taken at 2 minute intervals. The composition of the reaction mixture is given in the legend shown in each figure.

As previously reported (*1*), an induction period of several minutes' duration was always observed in acid media when the reaction was started by mixing the enzyme, substrate and ferrous ion simultaneously. In order to eliminate such induction periods, the ferrous salt was always added to the enzyme solution 10 minutes prior to the initiation of the reaction effected by adding the substrate. All the experiments were carried out at 30°.

Protein was assayed by the same method as described previously (*1*), and the pH of the reaction mixture was determined with a Beckman Model G pH-meter.

RESULTS

Effect of Enzyme Concentration—Fig. 1 shows the dependence of reaction rate on the enzyme concentration as measured at pH 6.8 in the presence of $2\times10^{-3}\,M$ of homogentisic acid and $5\times10^{-4}\,M$ of ferrous sulfate. As can

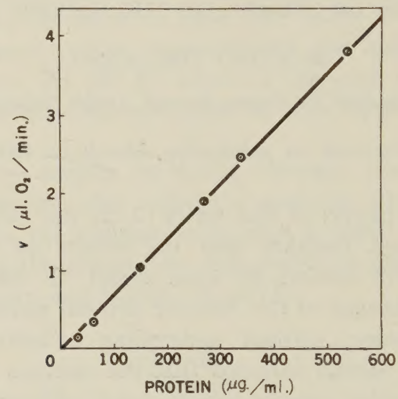

FIG. 1. Relationships between enzyme concentration and initial rate at a given concentration of substrate. Enzymatic activity was measured manometrically at 30°, pH 6.8. Each vessel contained enzyme solution, $2\times10^{-3}\,M$ of homogentisic acid, $5\times10^{-4}\,M$ of $FeSO_4$, $5\times10^{-2}\,M$ of phosphate buffer and distilled water to make a final volume of 2 ml. Gas phase was air.

be seen, the reaction rate was proportional to the enzyme concentration, at least up to 600 μg. of protein per ml., under these experimental conditions. Since it was feared that the diffusion of molecular oxygen into the liquid phase may become the rate-limiting factor at higher concentrations of the enzyme, all the experiments were carried out at enzyme concentrations lower than 600 μg. of protein per ml.

Effect of Substrate Concentration at Various pH's—It was reported in a previous paper (*1*) that ferrous ion inhibits the homogentisicase reaction at concentration higher than $1\times10^{-3}\,M$. It is also found that the substrate inhibits the reaction at concentrations higher than $1.4\times10^{-3}\,M$. In the present study, therefore, the effect of substrate concentration on the reaction rate has been studied at the substrate concentrations not exceeding $1.4\times10^{-3}\,M$; the ferrous ion concentration being fixed at $1\times10^{-3}\,M$. The experiments have been conducted at each side of pH 5.8, which has been found to be optimum for the reaction (*1*).

The data obtained at pH values higher than pH 5.8 are shown in Fig. 2 in which the reciprocal of rate, $1/v$*, is plotted against the reciprocal of substrate concentration, $1/[S]$. It will be seen that straight lines are obtained

at all the pH values examined as expected from the equation of Lineweaver and Burk (5). The fact that all the straight lines cut the ordinate at the same point indicated that the apparent maximum velocity, $V^{(Fe)}_{ap}$, is constant in the pH range tested. From the intercepts of these straight lines

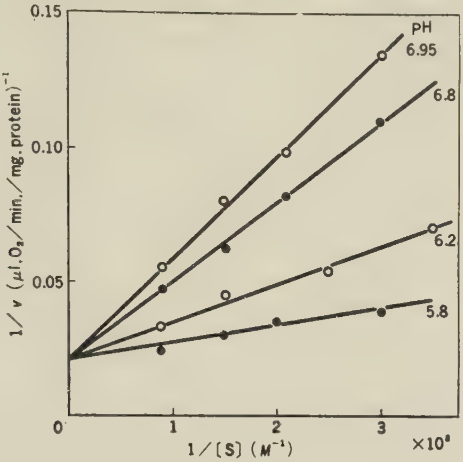

FIG. 2. Reciprocal of reaction velocity as a function of reciprocal of substrate concentration at various pH's around 6. Reaction velocity was measured manometrically at 30°. Each vessel contained the Fe^{++}-free enzyme, $1\times10^{-3}\,M$ $FeSO_4$, $3.3\times10^{-2}\,M$ phosphate buffer,the substrate, $1\times10^{-3}\,M$ glutathione and distilled water to make a final volume of 2 ml. Gas phase was air.

on the ordinate, it has been estimated that $V^{(Fe)}_{ap}$ has the value of 50 μl. O_2/minute/mg. protein. Since the slope of these straight lines increases with increasing pH values, it may be concluded that the substrate anion competes with the hydroxyl ion in combining with the enzyme molecule. That the substrate exists in this pH region in the form of an anion, due to the removal of a proton from its carboxyl group, may be inferred from its pK value ($pK=4.2$). From the slope of these straight lines, the apparent Michaelis constants, $K^{(S)}_{ap}$, were calculated, and on plotting these values against the reciprocal of hydrogen ion concentration, $1/[H^+]$, a linear relation was obtained as shown in Fig. 3.

Fig. 4. shows the Lineweaver-Burk plots of the data obtained at the acid side of pH 5.8. Linear relationships also exist in these cases. The straight lines, however, cut the ordinate at different points, and they fall on

* The symbols used in this paper have the following denotations: v, initial reaction rate; $V^{(Fe)}_{ap}$, reaction rate at a fixed concentration of ferrous ion and in the presence of excess substrate; $V^{(S)}_{ap}$, reaction rate at a fixed substrate concentration and in the presence of excess ferrous ion; Vm, hypothetical reaction rate if all the enzyme molecules were in the form of the active complex denoted by $EFe^{++}S$.

the same point on the abscissa. This fact indicates that the same value of $4.0\times10^{-4}\,M$ in these acid pH's, while $V_{ap}^{(Fe)}$ increases with increasing pH's. As shown in Fig. 5, a linear relationship seems to hold between $[H^+]$ and $V_{ap}^{(Fe)}$.

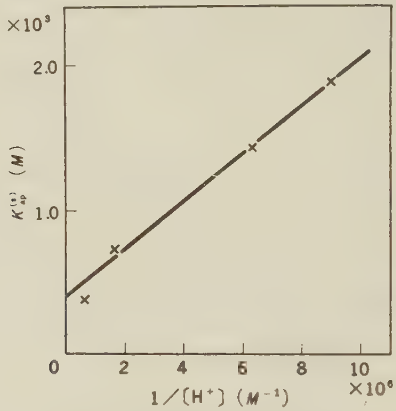

FIG. 3. Relationship between $K_{ap}^{(S)}$ and $1/[H^+]$.

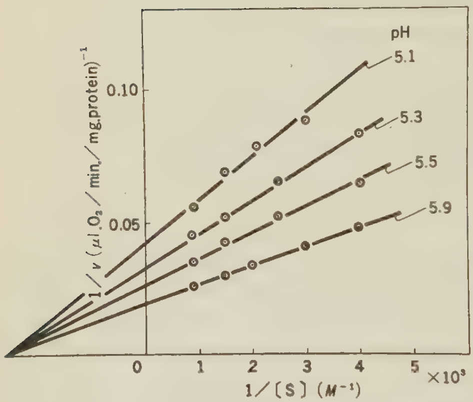

FIG. 4. Reciprocal of reaction velocity as a function of reciprocal of substrate concentration at various acidic pH's. All the conditions were the same as that described in Fig. 2.

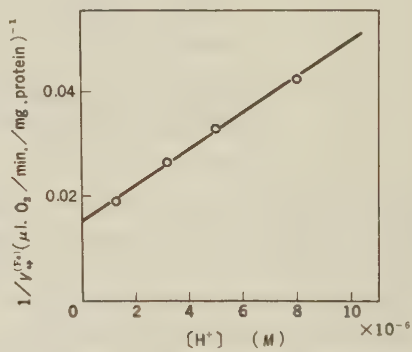

FIG. 5. Relationship between $1/V_{ap}^{(Fe)}$ and $[H^+]$.

Effect of Ferrous Ion Concentration at Various pH's—The effect of varying ferrous ion concentrations on the reaction rate with a fixed substrate concentration of $1.1\times10^{-3}\,M$ was studied at various pH values. When the reciprocal of rate, $1/v$, was plotted against the reciprocal of ferrous ion concentration, $1/[Fe^{++}]$, linear relationships were obtained at all the pH values examined as shown in Figs. 6 and 8. Fig. 6 shows that, at pH values higher than 5.8, the straight lines obtained at different pH's cut the abscissa at the same point, suggesting that the affinity of ferrous ion to the enzyme protein is not affected by pH in this pH region. The apparent maximum velocity,

$V_{ap}^{(S)}$, on the other hand, decreases with increasing pH value. A straight line is obtained when $1/V_{ap}^{(S)}$ is plotted against $1/[H^+]$ as shown in Fig. 7.

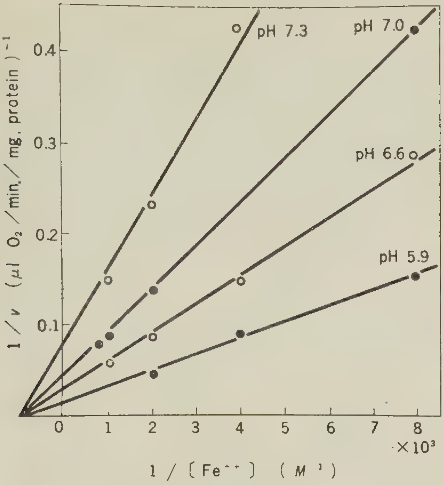

FIG. 6. The change of $1/v$ as a function of $1/[Fe^{++}]$ at various pH's around 6–7. Reaction veloctity was measured manometrically at 30°. Each vessel contained the Fe^{++}-free enzyme, $FeSO_4$, $1.1\times10^{-3}\,M$ homogentisic acid, $1\times10^{-3}\,M$ glutathione, $3.3\times10^{-2}\,M$ phosphate buffer and distilled water to make a final volume of 2 ml. Gas phase was air.

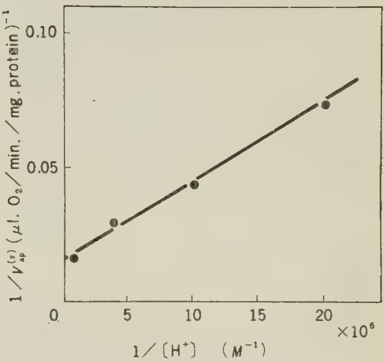

FIG. 7. Relationship between $1/V_{ap}^{(S)}$ and $1/[H^+]$.

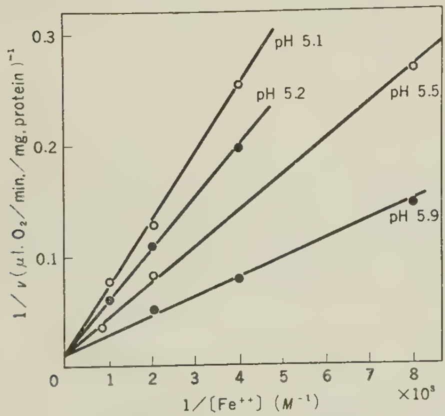

FIG. 8. Change of $1/v$ as a function of $1/[Fe^{++}]$ at various acidic pH's. All the conditions were the same as described in Fig. 5.

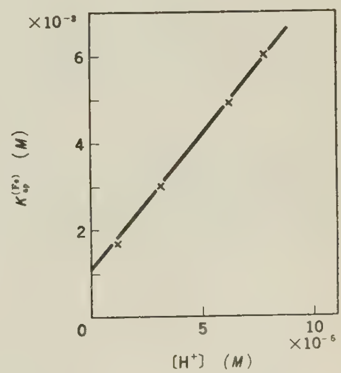

FIG. 9. Relationship between $K_{ap}^{(Fe)}$ and $[H^+]$.

The data plotted in Fig. 8 indicate that, in a more acid region than pH 5.8, ferrous ion competes with the hydrogen ion for the enzyme molecule. In fact, $V_{ap}^{(S)}$ has a constant value of 78 μl. O_2/minute/mg. protein at these acid pH's, and the slopes of the straight lines in Fig. 8 increase with decreas-

ing pH values. The apparent dissociation constant of the ferrous ion-enzyme complex, $K_{\mathrm{ap}}^{(Fe)}$, which was calculated from the slopes of the straight lines, bears a linear relationship to $[\mathrm{H}^+]$ as shown in Fig. 9.

Effect of Ferrous Ion Concentration on the 1/v vs. 1/[S] Relationships—In Fig. 10 are shown the $1/v$ vs. $1/[S]$ curves obtained at pH 6.1 in the presence of varying ferrous ion concentrations (1×10^{-3}, 5×10^{-4} and $3\times10^{-4}\,M$). It will be seen that the apparent Michaelis constant, $K_{\mathrm{ap}}^{(S)}$, has a value of $6.2\times10^{-3}\,M$ and it is not affected by ferrous ion concentration, whereas the apparent maximum velocity, $V_{\mathrm{ap}}^{(Fe)}$, increases with increasing ferrous ion concentration. A straight line is obtained when $1/V_{\mathrm{ap}}^{(Fe)}$ is plotted against $1/[Fe^{++}]$ as shown in Fig. 11.

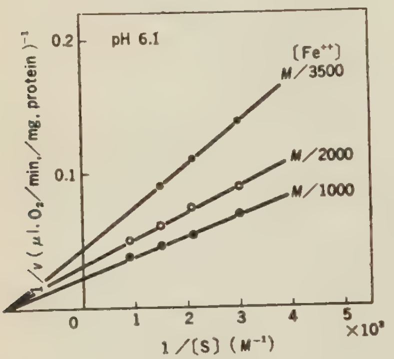

FIG. 10. The change of $1/v$ as a function of $1/[S]$ at various ferrous ion concentrations. Reaction velocity was measured manometrically at 30°. Each vessel contained the Fe^{++}-free enzyme, $FeSO_4$, homogentisic acid, $1\times10^{-3}\,M$ glutathione, $3.3\times10^{-2}\,M$ phosphate buffer and distilled water to make a final volume of 2 ml. Gas phase was air.

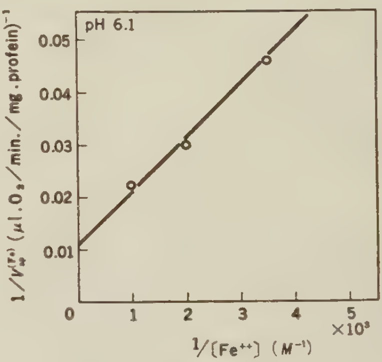

FIG. 11. Relationship between $1/V_{\mathrm{ap}}^{(Fe)}$ and $1/[Fe^{++}]$.

Mechanism of Enzyme Reaction—In order to interpret all the findings described above, the following set of equations may be proposed as representing the overall mechanism of the homogentisicase reaction:

$$\mathrm{E}^- + \mathrm{H}^+ \underset{}{\overset{K_1}{\rightleftharpoons}} \mathrm{E} \qquad (1)$$

$$\mathrm{E} + \mathrm{H}^+ \overset{K_2}{\rightleftharpoons} \mathrm{E}^+ \qquad (2)$$

$$\mathrm{E}^- + \mathrm{Fe}^{++} \overset{K_3}{\rightleftharpoons} \mathrm{E}^-\mathrm{Fe}^{++} \qquad (3)$$

$$\mathrm{E}^+ + \mathrm{S} \overset{K_4}{\rightleftharpoons} \mathrm{E}^+\mathrm{S} \qquad (4)$$

$$\mathrm{E} + \mathrm{Fe}^{++} \overset{K_3}{\rightleftharpoons} \mathrm{EFe}^{++} \qquad (5)$$

$$\mathrm{E} + \mathrm{S} \overset{K_4}{\rightleftharpoons} \mathrm{ES} \qquad (6)$$

$$\mathrm{EFe}^{++} + \mathrm{S} \overset{K_4}{\rightleftharpoons} \mathrm{EFe}^{++}\mathrm{S} \qquad (7)$$

$$ES + Fe^{++} \underset{k}{\overset{K_3}{\rightleftharpoons}} EFe^{++}S \qquad (8)$$

$$EFe^{++}S \longrightarrow EFe^{++} + P \qquad (9)$$

The possibility that the substrate-ferrous ion complex, $Fe^{++}S$, may act as the true substrate is not considered in the above scheme, since it was previously reported (*1*) that the progress of the reaction, in which the Fe^{++}-free enzyme was added to a preincubated mixture of ferrous ion and the substrate, was quite similar to that of the system in which the enzyme, ferrous ion and the substrate were simultaneously mixed.

Assuming that the reaction rate is proportional to the concentration of the complex, $EFe^{++}S$, and taking into consideration the fact that the enzyme concentration employed can be neglected as compared with the substrate and ferrous ion concentrations, the following equation can be derived from the above set of relations.

$$v=k\ [EFe^{++}S]$$

$$=\frac{ke}{\frac{K_3\ K_4}{[S][Fe^{++}]}+\frac{K_1\ K_3\ K_4}{[H^+][S][Fe^{++}]}+\frac{[H^+]\ K_3\ K_4}{K_2[S][Fe^{++}]}+\frac{K_1\ \ K_4}{[H^+][S]}+\frac{K_3[H^+]}{K_2[Fe^{++}]}+\frac{K_4}{[S]}+\frac{K_3}{[Fe^{++}]}+1} \qquad (10)$$

where e represents the total concentration of the enzyme, *i.e.*,

$$e=[E^-]+[E]+[E^+]+[E^-Fe^{++}]+[E^+S]+[ES]+[EFe^{++}]+[EFe^{++}S] \qquad (11)$$

Since the terms $\frac{[H^+]\ K_3\ K_4}{K_2\,[S]\,[Fe^{++}]}$ and $\frac{K_3\,[H^+]}{K_2\,[Fe^{++}]}$ may be neglected at the alkaline side of pH 5.8, Eq. 10 can be simplified to the following equation according to the procedure of Lineweaver and Burk;

$$1/v=(\frac{K_3}{[Fe^{++}]}+1)\ (\frac{K_1}{[H^+]}+1)\ \frac{K_4}{[S]}\cdot\frac{1}{Vm}+(\frac{K_3}{[Fe^{++}]}+1)\frac{1}{Vm} \qquad (12)$$

or

$$1/v=(\frac{K_4}{[S]}+\frac{K_1\ \ K_1}{[H^+]\ [S]}+1)\frac{K_3}{[Fe^{++}]}\cdot\frac{1}{Vm}+(\frac{K_4}{[S]}+\frac{K_1\ K_4}{[H^+]\ [S]}+1)\frac{1}{Vm} \qquad (13)$$

Since these two equations are in accordance with the experimental data presented in Figs. 2, 6 and 10, it will be easily deduced from Eqs. 12 and 13 that $K^{(S)}_{ap}$, $V^{(Fe)}_{ap}$ and $V^{(S)}_{ap}$ may be represented by Eqs. 14—16, respectively.

$$K^{(S)}_{ap}=(K_1/[H^+]+1)\ K_4 \qquad (14)$$

$$1/V^{(Fe)}_{ap}=(K_3/[Fe^{++}]+1)\ 1/Vm \qquad (15)$$

$$1/V^{(S)}_{ap}=\frac{K_1\ \ K_4}{[H^+]\ [S]}\cdot\frac{1}{Vm}+(K_4/[S]+1)\ 1/Vm \qquad (16)$$

The validity of these equations seems to be proved by the linear relationships indicated in Figs. 3, 7 and 11.

By the analysis of the data presented in Fig. 3 representing Eq. 14, K_4 and K_1 can be shown to have the values $4.0\times10^{-4}\,M$ and $4.2\times10^{-7}\,M$, respectively (K_4 from the intercept on the ordinate and K_1 from the slope divided by K_4). From the intercept on the abscissa in Fig. 6, K_3 is calculated

according to Eq. 13 to be $1.2\times10^{-3}\,M$. As already stated, $V_{\mathrm{ap}}^{(Fe)}$ has been experimentally estimated from the data given in Fig. 2 to be 50 μl. O_2/minute/mg. protein. By introducing the values of K_3 and $V_{\mathrm{ap}}^{(Fe)}$ into Eq. 15, it is deduced that V_m has a value of 110 μl. O_2/minute/mg. protein.

The straight line in Fig. 7 represents Eq. 16. By a similar analysis of the date presented in Fig. 7, the values of K_1 and V_m are again computed to be $4.7\times10^{-7}\,M$ and 90 μl. O_2/minute/mg. protein, respectively. The value of K_4 can be further checked by graphically determining the value of $K_{\mathrm{ap}}^{(S)}$ from Fig. 10 and introducing it into Eq. 14. This gives $4.2\times10^{-4}\,M$ as the value of K_4. By applying the data given in Fig. 11 to Eq. 15, K_3 and V_m are again calculated to be $1.1\times10^{-3}\,M$ and 100 μl. O_2/minute/mg. protein, respectively.

For the acid side of pH 5.8, Eq. 10 can be rewritten in the following simplified reciprocal form by neglecting the terms $\frac{K_1\,K_3\,K_4}{[H^+][S][Fe^{++}]}$ and $\frac{K_1\,K_4}{[H^+][S]}$.

$$1/v=\left(\frac{K_3}{[Fe^{++}]}+\frac{K_3\,[H^+]}{K_2\,[Fe^{++}]}+1\right)\frac{K_4}{[S]}\cdot\frac{1}{Vm}+\left(\frac{K_3}{[Fe^{++}]}+\frac{K_3\,[H^+]}{K_2\,[Fe^{++}]}+1\right)\frac{1}{Vm} \tag{17}$$

or

$$1/v=\left(\frac{[H^+]}{K_2}+1\right)\left(\frac{K_4}{[S]}+1\right)\frac{K_3}{[Fe^{++}]}\cdot\frac{1}{Vm}+\left(\frac{K_4}{[S]}+1\right)\frac{1}{Vm} \tag{18}$$

The data given in Figs. 4 and 8 are in accordance with Eqs. 18 and 19, respectively. The apparent Michaelis constant obtainable from the intercept on the abscissa in Fig. 4, therefore, corresponds to K_4 according to Eq. 17, and it has a value of $4.0\times10^{-4}\,M$. Furthermore, Eqs. 17 and 18 show that $V_{\mathrm{ap}}^{(Fe)}$, $V_{\mathrm{ap}}^{(S)}$ and $K_{\mathrm{ap}}^{(Fe)}$ can be written as follows:

$$V_{\mathrm{ap}}^{(Fe)}=\left(\frac{K_3}{[Fe^{++}]}+\frac{K_3\,[H^+]}{K_2\,[Fe^{++}]}+1\right)\frac{1}{Vm} \tag{19}$$

$$V_{\mathrm{ap}}^{(S)}=\left(\frac{K_4}{[S]}+1\right)\frac{1}{Vm} \tag{20}$$

$$K_{\mathrm{ap}}^{(Fe)}=\left(\frac{[H^+]}{K_2}+1\right)K_3 \tag{21}$$

Eqs. 19 and 21 are represented by the straight lines drawn in Figs. 5 and 9, respectively. By applying Eq. 19 to the data shown in Fig. 5, K_2 and V_m are calculated to be $2.7\times10^{-6}\,M$ and 120 μl. O_2/minute/mg. protein, respectively. By introducing the values of $V_{\mathrm{ap}}^{(S)}$ obtained from the data in Fig. 8

TABLE I

Values of pK's and Vm at 30°

pK_1	6.4–6.5
pK_2	5.6–5.7
pK_3	2.9–3.0
pK_4	3.4
V_m	*ca.* 100 μl. O_2/minute/mg. protein

into Eq. 20, V_m is again computed to be 100 μl. O_2/minute/mg. protein. The analysis of the data shown in Fig. 9 according to Eq. 21 gives the values of $2.0\times10^{-6}\,M$ for K_2 and $1.1\times10^{-3}\,M$ for K_3, respectively.

As described above, the results of analysis from different angles of the kinetic data give closely similar or identical values for K as well as for V_m, indicating the correctness of the assumptions made in Eqs. 1–9. The values of pK's and V_m obtained above are summarized in Table I.

DISCUSSION

As suggested in the preceding papers (*1*, *2*), homogentisicase appears to possess at least two types of dissociable groups which are essential for the enzyme activity, namely, the SH group and the phenolic OH group. The substrate seems to combine with the SH group and to have a close relation to the phenolic OH group. The ferrous ion, on the other hand, is assumed to associate with some negatively charged group and presumably connected, in some way, to the phenolic OH group.

The results of the kinetic studies reported in the present paper suggest that a dissociable group, pK of which has a value of 6.4–6.5, is responsible for the substrate combination. The nature of this group can not yet be identified from the experimental findings available to date. In view of the fact that a SH group is concerned with the linkage of the substrate with the enzyme molecule, it is tempting to attribute the SH group to the dissociable group in question. However, since the pK value of the SH group of cysteine residue is much higher than 6.4–6.5, this assumption does not seem tenable. It has been known that SH groups of thiophenols and dehydrocysteine residues, *i. e.* –SH and $\underset{\text{-OC-C-NH-}}{\overset{\text{CH-SH}}{\|}}$, possess lower pK values, comparable to pK of the dissociable group in question (in the case of thiopenol, pK= 7.2). Therefore, the possibility that such special SH groups might be involved can not be excluded, though the existence of such groups in proteins has not yet been reported. The other possibility is that the dissociable group may be an imidazole group pK of which might be, for some reason, higher than its usual value (*5*. *7*).

It may be more safely inferred that the another dissociable group having a pK value of 5.6–5.7 is the imidazole group of histidine residue. Available evidence indicates that this group is the site of ferrous ion binding.

The analysis of kinetic data given in the present paper provides no evidence for the possibility that the ferrous ion bound to the enzyme protein acts as a bridge in the enzyme-substrate complex. This suggests that the complexes referred to as E^+S and ES are actually formed as given in Eqs. 4 and 6, respectively. It can also be excluded by kinetic analysis that the combination reaction between the substrate and the enzyme is influenced by the attachment of ferrous ion to the latter.

The role of the phenolic OH group in the enzyme reaction is rather

difficult to explain. The fact that the OH group is irreversibly inactivated by tyrosinase and this inactivation can be completely protected by either the substrate of ferrous ion suggests that the OH group is in some way related to the combination of both the substrate and ferrous ion with the enzyme molecule. It is, for example, likely that the OH group is located very close to both the active SH and imidazole group with which the substrate and ferrous ion combine, respectively. It also seems probable that the OH group itself binds to neither the substrate nor the ferrous ion, but is essential for the maintenance of the reactive structure of the whole enzyme molecule. With such assumption, the experimental results concerning the OH group seem easily explainable.

SUMMARY

1. In continuation of the studies reported earlier, the action of homogentisicase isolated from beef liver was investigated in detail from a kinetic point of view. The effects of hydrogen ion, substrate and ferrous ion concentrations on the enzyme activity were studied in the pH range from 5.0 to 7.0.

2. By analysis of the results obtained, it was inferred that in the alkaline side of pH 5.8 the hydroxyl ion competed with the substrate anion for the enzyme molecule and in the acid side hydrogen ion competes with the ferrous ion. The substrate seemed to associate with an acid group of the enzyme protein, and *pK* of this group was determined to be 6.5 at 30°. The ferrous ion seemed to associate with a basic group, *pK* of which had a value of 5.7 at 30°.

3. The Michaelis constant and dissociation constant of the ferrous ion-enzyme complex were obtained as the values of 4.0×10^{-4} and $1.2\times10^{-3}\,M$, respectively, at 30°.

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STUDIES ON ACTIVE GROUPS OF PAPAIN

II. INHIBITION OF CYANIDE-ACTIVATED PAPAIN BY DIISOPROPYL FLUOROPHOSPHATE*

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Recently it has been reported that endopeptidases, such as trypsin (*1*, *2*), chymotrypsin (*1*, *2*), and proteinase of *Bacillus subtilis* N′ (*3*), and some esterases (*4*, *5*, *6*) are strongly inhibited by diisopropyl fluorophosphate (DFP). One mole of this reagent per mole of enzyme causes complete inhibition. *O*-phosphoserine has been obtained from the acid or enzymatic hydrolysates of the diisopropyl phosphoryl derivatives (*7–10*).

However, no effect of DFP on untreated or cysteine-activated papain was observed by Jansen *et al.* (*5*) and Kimmel and Smith (*11*). This difference in behaviour towards DFP is very interesting, since papain also has an endopeptidatic action.

In the preceding paper (*12*), it was reported that the inhibition of papain by aldehyde reagents depends on the activators. Untreated papain and the cyanide-activated form were inhibited by aldehyde reagents but the cysteine-, thioglycolate-, and hydrogen sulfide-activated papains were not affected.

In the present investigation, it was found that after activation by cyanide papain was inhibited by DFP. This reagent did not inhibit cysteine-, thioglycolate-, and hydrogen sulfide-activated papains. Mercuripapain and untreated enzyme were not affected by DFP but were inhibited by aldehyde reagents. A stable DFP-inhibited papain, containing one atom of phosphorus per mole of papain, was obtained in crystalline form. A phosphopeptide was separated from the partial hydrolysate of DFP-inhibited papain by fractionation with Dowex 50 chromatography. After complete hydrolysis of the phosphopeptide by 4 *N* HCl, component amino acids were identified by paper chromatography.

EXPERIMENTAL

Crystalline Papain—As described in the preceding paper (*12*), crystalline papain was prepared from dried papaya latex using a slight modification of the method of Kimmel and Smith (*11*).

* An outline of this work was given at the 9th Meeting of the Symposia on the Structure of Proteins in Osaka, in November, 1958.

Mercuripapain—Mercuripapain was prepared from twice recrystallized papain according to the method of Kimmel and Smith. (*11*).

Proteinase of Bacillus subtilis N'—Crystalline material was kindly supplied by Nagase & Co., Ltd., Osaka.

Diisopropyl Fluorophosphate—This compound was kindly supplied by Dr. T. Ikenaka of the Akabori laboratory, University of Osaka.

Assay for Proteolytic Activity of Papain—As described in the preceding paper (*12*), the proteolytic activity of papain was determined by a method essentially based on the methods of Anson (*13*) and Kunitz (*14*).

Determination of Phosphorus—Determination of phosphorus was carried out according to the method of Dryer *et al.* (*15*), in which *N*-phenyl-*p*-phenylenediamine* is used as reducing agent.

Preparation and Crystallization of DFP-Inhibited Papain—Crystalline DFP-inhibited papain was prepared from twice recrystallized papain according to the following procedure.

Twice recrystallized papain was dialyzed against 0.1 *M* phosphate buffer, pH 7.4, to dissolve it in minimal volume of buffer solution. The enzyme solution was mixed with one tenth of its volume of potassium cyanide (adjusted to pH 7.4). The mixture was incubated at 30° for 3 hours. The cyanide-activated papain was mixed with an excess of DFP, and incubated at 30° for 20 minutes. After addition of DFP, the pH of the mixture was adjusted to 7.4, because formation of hydrogen fluoride caused an increase in acidity. The mixture was dialyzed for 24 hours against deionized water and the centrifuged at $1{,}500 \times g$ to remove insoluble material. The supernatant was precipitated with 0.4 saturated ammonium sulfate. The turbid solution allowed to stand at 4° for 16 hours, and then centrifuged at $15{,}000 \times g$. The precipitate thus obtained was

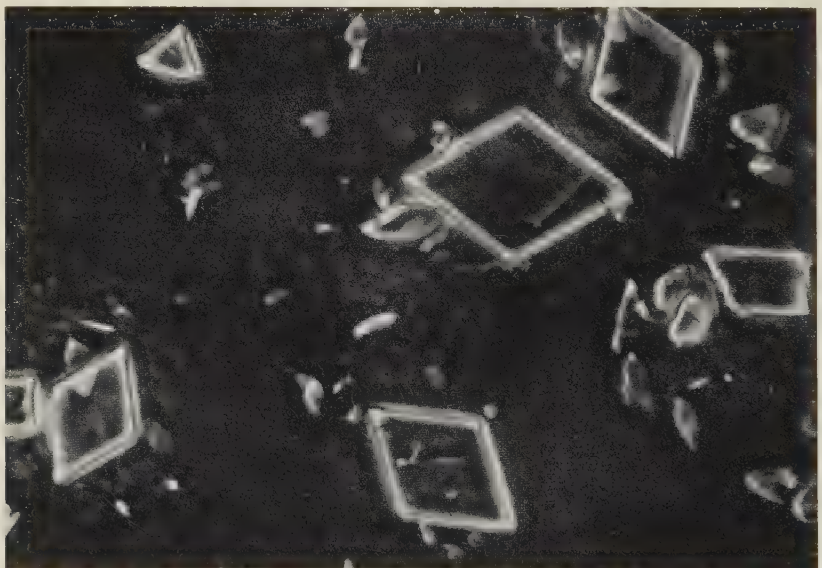

FIG. 1. Crystals of DFP-inhibited papain. ×200

* This compound was kindly supplied by Dr. M. L. Huggins of Eastman Kodak Co., Ltd., New York.

suspended in a small volume of deionized water, and dialyzed against deionized water. From the dialysate, DEP-inhibited papain was crystallized on addition of small amounts of sodium chloride. Recrystallization was carried out by the same procedure. As shown in Fig. 1, crystals of DFP-inhibited papain are rhomboidal plates. Although the shape of crystals of papain prepared by Balls and Lineweaver (*16*) is not rhomboidal but a long hexagonal form, crystals of untreated papain prepared in our laboratory have the same shape as those of the DFP-inhibited enzyme. It is uncertain what causes the difference in shape of the crystals but long hexagonal crystals were prepared from the cyanide-activated form and rhomboidal crystals from untreated enzyme. The phosphorus content of DFP-inhibited papain was estimated at 0.95 moles per mole of papain, assuming the molecular weight of papain to be 20,700 (*17*).

Partial Hydrolysis of DFP-Inhibited Papain and Separation of Phosphopeptide—DFP-inhibited papain (200 mg.) was denatured by 2 N HCl and suspended in 40 ml. of 0.1 *M* Tris buffer, pH 7.4, after centrifugation and neutralization with sodium hydroxide. Approximately 10 mg. of trypsin was added to the suspension and incubated at 30° for 16 hours. One second volume of 6 *N* HCI was added to the mixture and the resulted precipitate was removed by centrifugation. The mixture was repeatedly evaporated to remove hydrochloric acid.

The hydrolysate was further hydrolyzed in a sealed tube with 2 *N* HCl by heating to 105° for 23 hours. After removal of hydrochloric acid by repeated evaporation, phosphopeptide was separated by fractionation with H-form of Dowex 50 (200-350 mesh) chromatography according to the method of Schaffer *et al.* (*8*).

Identification of Amino Acids—Component amino acids of the phosphopeptide were identified by paper chromatography of dinitrophenyl derivatives according to the method of Levy (*18*).

RESULTS

As shown in Fig. 2, papain was strongly inhibited by DFP after activation by cyanide. The amount of DFP required for complete inhibition of cyanide-activated papain is considerably in excess of the enzyme and this inhibition thus differs from the stoichiometrical relation with the proteinase of *Bac. subtilis* N′ (*3*) or with chymotrypsin (*1*). DFP-inhibited papain was not reactivated by addition of cysteine or cyanide after dialysis. Although Jansen *et al.* (*5*) and Kimmel and Smith (*11*) have reported that untreated papain and cysteine-activated papain are not affected by DFP, the reports are not in conflict with the inhibition of cyanide-activated enzyme by DFP, since the cysteine-activated and untreated papains prepared in our laboratory were also not inhibited by DFP (Table I and II).

As shown in Table III, cyanide-activated papain was protected from DFP-inhibition with simultaneous or prior addition of cysteine to DFP. The molar concentration of cysteine required for complete protection was approximately equal to that of DFP.

To examine the possibility that cysteine reacts directly with DFP and removes it from reaction system, the effect of cysteine on the inhibition of proteinase of *Bac. subtilis* N′ by DFP was investigated. The reaction of DFP with the proteinase of *Bac. subtilis* N′ was not affected by cysteine (Table IV).

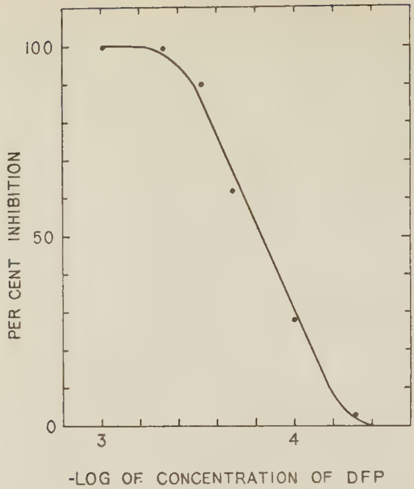

FIG. 2. Inhibition of papain by DFP. Enzyme concentration ; 0.0102 per cent. 0.1 *M* phosphate buffer, pH 7.4. Substrate ; 0.5 per cent casein. Enzyme was activated with 0.25 *M* potassium cyanide and then added with DFP. After 5 minutes incubation, activity was assayed.

TABLE I

Effect of DFP on Untreated Papain

Enzyme concentration ; 0.0029 per cent. 0.1 *M* phosphate buffer, pH 7.4. After enzyme had been incubated with DFP for 10 minutes cysteine was added and activity was assayed.

DFP	Cysteine	Activity
—	—	.000
—	5×10^{-3} *M*	.291
5×10^{-4} *M*	—	.029
,,	5×10^{-3} *M*	.250

TABLE II

Effect of DFP on Cysteine-Activated Papain

Enzyme concentration ; 0.0235 per cent in Exp. 1 and 0.0185 per cent in Exp. 2. 0.1 *M* phosphate buffer, pH 7.4. After enzyme had been incubated with cysteine for 10 minutes, DFP was added to the mixture and activity was assayed.

Exp. No.	DFP	Cysteine	Activity
1	—	5×10^{-3} *M*	1.130
	5×10^{-4} *M*	,,	1.102
2	—	,,	1.179
	5×10^{-4} *M*	,,	1.207

It appears, therefore, that cysteine reacts with cyanide-activated papain and makes it unsusceptible to DFP. Like the cysteine-activated papain, hydrogen sulfide- and thioglycolate-activated papains were hardly inhibited by DFP (Table V).

TABLE III

Effect of Cysteine on DFP-Inhibition of Cyanide-Activated Papain

Enzyme concentration; 0.006 per cent. 0.1 *M* phoshate buffer, pH 7.4. Enzyme was activated with 0.1 *M* potassium cyanide (adjusted to pH 7.4) for 40 minutes, and then cysteine and DFP were added to the mixture. After 10 minutes, activity was assayed.

DFP	Cysteine	Activity
—	—	.627
5×10^{-4} *M*	—	.065
—	2.5×10^{-3} *M*	.765
5×10^{-4} *M*	,,	.775
,,	2.5×10^{-4} *M*	.721
5×10^{-5} *M*	,,	.775

TABLE IV

Effect of Cysteine on DFP-Inhibition of Bacillus subtilis N′

Enzyme concentration; 0.0105 per cent. 0.1 *M* phosphate buffer, pH 7.4. Enzyme was added with cysteine and DFP. After the mixture had been incubated at 30° for 5 minutes, activity was assayed.

DFP	Cysteine	Activity
—	—	1.440
5×10^{-4} *M*	—	.034
—	2.5×10^{-3} *M*	1.479
5×10^{-4} *M*	,,	.042
,,	5×10^{-3} *M*	.076

The DFP-inhibition of mercuripapain differs from that of aldehyde reagents. Like the untreated papain, mercuripapain was not affected by DFP (Table VI). However, when mercuripapain was activated with cyanide before the addition of DFP, it was potently inhibited and this inhibition could be prevented by the simultaneous addition of cysteine. From these results, it is concluded that activation with cyanide is necessary for the inhibition of papain by DFP. To confirm this conclusion, the quantitative relation between activation by cyanide and inhibition by DFP was investigated (Table VII). Papain was partially activated by cyanide and then DFP was added. After 5 minutes, cysteine was added to the mixture and then the activity was assayed. The enzyme which was not activated by cyanide and

TABLE V

Effect of DEP on Thioglycolate- and H_2S-Activated Papains

Enzyme concentration; 0.0235 per cent in Exp. A-1, 0.019 per cent in Exp. A-2, 0.0402 per cent in Exp. B-1, and 0.0253 per cent in Exp. B-2, respectively. In Exp. A, enzyme was activated with thioglycolate for 10 minutes and then added with DFP. After 5 minutes, activity was assayed.

Exp. No.	DFP	Activator	Activity
A-1	—	$5\times10^{-3}\,M$ TG[1)]	.590
	$5\times10^{-4}\,M$	,,	.611
A-2	—	$2.5\times10^{-2}\,M$ TG[1)]	.578
	$5\times10^{-4}\,M$	,,	.578
B-1	—	H_2S[2)]	.485
	$5\times10^{-4}\,M$	,,	.427
B-2	—	H_2S[3)]	.437
	$5\times10^{-4}\,M$	,,	.441

1) Thioglycolate was diluted with $0.1\,M$ phosphate buffer (pH 7.4)
2) Enzyme solution was bubbled with hydrogen sulfide for 20 minutes.
3) Enzyme solution was bubbled with hydrogen sulfide for 5 minutes.

TABLE VI

Effect of DFP on Mercuripapain

Enzyme concentration; 0.0045 per cent in Exp. 1 and 0.0032 per cent in Exp. 2. $0.1\,M$ phosphate buffer (pH 7.4) was used in Exp. 1 and $0.05\,M$ Tris buffer (pH 7.4) in Exp. 2. Enzyme was incubated with DFP for 20 minutes at 30° and then activated with cysteine. After 5 minutes activity was assayed.

Exp. No.	DFP	Cysteine	Activity
1	—	$5\times10^{-3}\,M$	.934
	$5\times10^{-4}\,M$	—	.000
	,,	$5\times10^{-3}\,M$	1.030
2	—	$2.5\times10^{-3}\,M$	.777
	$5\times10^{-4}\,M$	—	.010
	,,	$2.5\times10^{-3}\,M$	.747

remained in the inactive form should be uninhibited by DFP. The sum of the activities of the partially activated enzyme (a) and of the uninhibited enzyme (b) are shown in the right column (a+b) of Table VII. They are approximately constant, ranging from 1.279 to 1.336. Therefore it can be considered that activation of papain by cyanide is indispensable for inhibition by DFP.

TABLE VII

Quantitative Relation between Cyanide-Activation and DFP-Inhibition

Enzyme concentration; 0.018 per cent. 0.1 M phosphate buffer, pH 7.4. In series a, enzyme was activated with cyanide for 10 minutes and then activity was assayed. In series b, enzyme was activated with cyanide for 10 minutes and then incubated with DFP for 5 minutes. After addition of cysteine, activity was assayed.

Exp. No.		Reagent added			Activity	a+b
		KCN	DFP	Cysteine		
1	a	$5\times10^{-3}\,M$	—	—	.345	
	b	,,	$10^{-3}\,M$	$5\times10^{-3}\,M$	.934	1.279
	a	$1.5\times10^{-2}\,M$	—	—	.537	
	b	,,	$10^{-3}\,M$	$5\times10^{-3}\,M$	.753	1.290
	a	$2.5\times10^{-2}\,M$	—	—	.660	
	b	,,	$10^{-3}\,M$	$5\times10^{-3}\,M$	.662	1.322
	a	$5\times10^{-2}\,M$	—	—	.809	
	b	,,	$10^{-3}\,M$	$5\times10^{-3}\,M$	.527	1.336
2		—	—	$5\times10^{-3}\,M$	1.211	

As shown in Table VIII, sodium bisulfite-treated papain was further inhibited by DFP. In Exp. 1, after incubation with DFP, bisulfite-treated

TABLE VIII

Effect of DFP on Bisulfite-Treated Papain

Enzyme concentration; 0.063 per cent in Exp. 1, 0.022 per cent in Exp. 2, and 0.0148 per cent in Exp. 3. 0.03 M Tris buffer, pH 7.4, in Exp. 1 and 0.1 M phosphate buffer, pH 7.4, in Exp. 2 and 3. Enzyme was incubated with sodium bisulfite for 60 minutes and then added with DFP. After 10 minutes incubation, cysteine was added to the mixture. After 5 minutes activity was assayed. In Exp. 1, activity was determined on aliquots removed from the original reaction mixture containing each component in high concentration for determination of phosphorus.

Exp. No.	$NaHSO_3$	DFP	Activity
1.	—	—	1.620
	$6.6\times10^{-3}\,M$	—	1.092
	,,	$8.3\times10^{-3}\,M$	.488
2.	—	—	1.188
	$5\times10^{-3}\,M$	—	.229
	,,	$5\times10^{-4}\,M$	.146
3.	—	—	1.018
	$5\times10^{-3}\,M$	—	.263
	,,	$2.5\times10^{-4}\,M$	.148

papain was precipitated and washed with 0.22 *M* trichloroacetic acid. There were 0.65 moles of phosphorus per mole of enzyme in the ashed precipitate.

A phosphopeptide was obtained from the partial hydrolysate of DFP-inhibited papain by Dowex 50 chromatography (Fig. 3). Peak A was inorganic phosphate and peak B and C were phosphopeptides. Peak B was not further

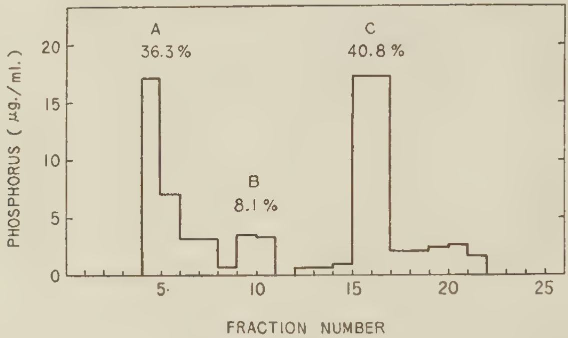

FIG. 3. Dowex 50 chromatogram of partial hydrolysate of DFP-inhibited papain. Material hydrolyzed by trypsin and 2 *N* HCl. 200–350 mesh resin in hydrogen form. Column 2×6 cm. Eluent 0.05 *N* HCl. Fraction size 2 ml.

analyzed because of lack of the amount needed for identification of component amino acids. After complete hydrolysis of the sample of peak C with 4 *N* HCl by heating to 105° for 24 hours, dinitrophenyl derivatives of the sample were prepared according to the method of Sanger (*19*).

After extraction with ether dinitrophenyl amino acids in the ether phase were identified by two-dimensional paper chromatography, while no dinitrophenyl amino acid was found in the aqueous layer. 1.1 moles of glycine, 0.9 mole of alanine, 0.9 mole of serine and 1.0 mole of acidic amino acid per atom of phosphorus were found in the phosphopeptide. They are very similar to the component amino acids of a phosphopeptide obtained from DIP-trypsin or DIP-proteinase of *Bac. subtilis N'*. Acidic amino acid could not be determined to be glutamic acid or aspartic acid, because of lack of amount needed for further analysis. It is uncertain which amino acid is bound with diisopropyl phosphoryl group (DIP).

DISCUSSION

The present results indicate that only cyanide-activated papain is inhibited by DFP. Untreated papain and the cysteine-activated form were not affected, as reported by Jansen *et al.* (*5*) and Kimmel and Smith (*11*). As shown in Table VII, the activation of papain by cyanide is indispensable for inhibition by DFP.

DFP-inhibited papain was very stable and could be obtained in crystalline form. This DFP-inhibited papain was not reactivated by addition of

activators after dialysis.

Bisulfite-treated papain was further inhibited by DFP and phosphorus was found in the enzyme protein after DFP-treatment and precipitation by trichloroacetic acid. This suggests that the hydroxy group of cyanohydrin acts as an acceptor for the diisopropyl phosphoryl group, since aldehyde reacts with sodium bisulfite to give an addition compound which is structurally similar to cyanohydrin.

As discussed in the preceding paper (*12*), it is conceivable that there are two groups, that is, an aldehyde and a sulfhydryl group, close to each other in the active area of papain. The following result may support the above hypothesis. Although in papain itself 0.96 sulfhydryl groups per enzyme molecule were determined by Boyer's method (*20*), 0.38 and 0.57 sulfhydryl groups were estimated in DFP-inhibited and hydroxylamine-inhibited papain, respectively. The reaction of *p*-chloromercuric benzoate with the sulfhydryl groups of papain may be sterically hindered by introduction of DFP or hydroxylamine.

In cyanide-activated papain, the hydroxy group of cyanohydrin which is converted from the aldehyde group may act as one of the active sites instead of the sulfhydryl group in cysteine- or thioglycolate-activated papain. The difference between the action of the cysteine- or thioglycolate-activated papain and the cyanide-activated enzyme may depend on a difference in the active group.

On the other hand, there remains another possibility that a sulfhydryl and an aldehyde group affect indirectly proteolytic action of the enzyme, since the preliminary results for analysis of phosphopeptide suggest a resemblance in structure of active site between papain and trypsin.

SUMMARY

1. The effect of DFP on several activated forms of papain was investigated. It was found that only cyanide-activated papain was completely inhibited by DFP.

2. Cysteine-, thioglycolate-, and hydrogen sulfide-activated papains and mercuripapain were not affected by DFP.

3. Bisulfite-treated papain was further inhibited by DFP. Phosphorus was present in the enzyme protein after treatment with DFP and precipitation by trichloroacetic acid.

4. Papain was as strongly inhibited by DFP as it was activated with cyanide.

5. A phosphopeptide was separated from partial hydrolysate of DFP-inhibited papain by fractionation with Dowex 50 chromatography. After complete hydrolysis of the phosphopeptide by 4 N HCl, glycine, alanine, serine, and acidic amino acid were identified by paper chromatography.

6. The existence of two groups, namely an aldehyde and a sulfhydryl group, in the active area of papain and the difference between cyanide-activation and cysteine-, thioglycolate- or hydrogen sulfide-activation are discussed.

The author wishes to express his gratitude to Prof. K. Okunuki and Prof. S. Akabori for their kind support in this work.

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STUDIES ON THE NEW GLYCOLIPIDE IN OYSTER

V. ON THE NITROGENOUS COMPONENTS AND STRUCTURE OF THE GLYCOLIPIDE

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(Received for publication, May 4, 1959)

In a previous paper, Akiya and Nakazawa reported the isolation of a new glycolipide (*1*) from the soft tissues of oyster. Further investigation of this glycolipide revealed that it has a structure different from the known types of cerebroside and contains a sugar portion consisting of a trisaccharide, D-glucopyranosyl$<$ 4.1 $>$-D-glucopyranosyl-$<$ 4.1 $>$-L-fucopyranose, which is combined with the other part of the glycolipide at its terminal reducing group forming a glycosidic linkage (*2*).

On the other hand, the fatty acid moiety of this glycolipide was identified as 14-methyl-4-pentadecenoic acid (*3*, 4).

In the present paper, the results of a further study of the other structural parts of the glycolipide, together with several observations which led to its proposed total structure will be reported.

After hydrolysis of this glycolipide, $C_{44}H_{82}O_{21}N_2S$ (Fig. 1-(J) in the first paper of this series (*1*)), with hydrochloric acid and removal of fatty acid from the hydrolysate with petroleum ether, lactic acid was detected in ether extract of the aqueous layer. Further the aqueous layer after the above ether extraction gave a basic compound which was isolated as its reineckate and converted into a picrate to identify with the authentic specimen of the same salt of choline.

On addition of mercuric acetate, the filtrate obtained after removal of the choline reineckate and excess of the reagent furnished the mercuric salt of taurine.

Thus, the starting glycolipide was proved to contain three components, lactic acid, choline and taurine in its total structure besides trisaccharide and fatty acid portions.

Quantitative estimations of these three new components as well as of the fatty acid were performed and the results are given in Table I.

From the data shown in Table I, it is evident that the glycolipide consists of each one mole of trisaccharide, fatty acid, choline, taurine and lactic acid.

An aqueous solution of this glycolipide was passed through columns of

Table I

Results of Determination of the Components Contained in the Glycolipide

	Calcd. for $C_{44}H_{82}O_{21}N_2S$	Found	Molar ratio
	%	%	
Choline	10.33	10.05	0.97
Taurine	12.43	13.11	1.05
Fatty acid	25.25	26.91	1.06
Lactic acid	8.94	7.63	0.85
Trisaccharide[1)]	48.40	45.90	0.95

1) This was reported in the second paper of this series (*2*)

Amberlite IR 120 (H^+–type) (A), and of Amberlite IRA 410 (OH^-–type) (B) successively. Elution of each column with aqueous 0.1 *N* hydrochloric acid (Solvent 1), followed by 80% ethanolic 0.1 *N* hydrochloric acid (Solvent 2), gave four effluents A_1, B_1, A_2, and B_2*. After evaporation of the solvents, each effluent gave the decomposition products of the glycolipide which were further heated with 10 per cent hydrochloric acid to complete hydrolysis (the hydrolysates thus obtained from A_1, A_2, B_1 and B_2 will be represented with A'_1, A'_2, B'_1 and B'_2 respectively). Choline could be detected in the hydrolysate A'_2 by paper chromatography. The water-insoluble precipitate appearing in this hydrolysate was assumed to be fatty acid from its solubilities in alkaline solution and ether. No choline and fatty acid were found in the hydrolysate A'_1.

On the other hand, taurine and lactic acid were identified in both hydrolysates B'_1 and B'_2. Furthermore, these hydrolysates gave positive Molisch's reaction which was not the case for the hydrolysates A'_1 and A'_2. Therefore, the carbohydrates must be contained in the hydrolysates B'_1 and B'_2.

In another run of decomposition of the glycolipide with Amberlite IR 120 and IRA 410, the effluents, A_2 and B_2, were collected by elution of each column with Solvent 2. The effluent obtained from the column of Amberlite IR 120 furnished cationic component. This component gave a picrate (m.p. 95–6°) which was assumed to be a picrate of *O*-(14-methyl-4-pentadecenoyl)-choline from its analysis.

Paper chromatography** of the component gave only one spot with R_f value 0.85 giving a coloration different from that of choline (R_f 0.28) with Dragendorff's reagent. The area of the spot with R_f value 0.85 on this paper chromatogram was cut out, and the cutting was extracted with aqueous ethanol. After heating with 10 per cent hydrochloric acid and

* A_1, B_1, A_2 and B_2 represent the corresponding effluents obtained from column A and B with Solvent 1 and 2 respectively and vice versa.

** A solvent system butanol-acetic acid-H_2O (25 : 6 : 25) was used.

examination of the hydrolysate by paper chromatography with the same solvent, choline was detected.

From the column of Amerberlite IRA 410, anionic component was eluted and was tested by paper chromatography*. No spot corresponding to taurine (R_f 0.43), but a new spot (R_f 0.25) which showed slight coloration with ninhydrin reagent and gave positive reaction with ammoniacal silver nitrate was detected. And on alkaline hydrolysis of this component, an acidic substance which gave a spot with R_f 0.77** was obtained. On further heating of this substance with 1 *N* hydrochloric acid taurine and lactic acid were obtained. This new intermediate giving the spot with R_f value 0.77 was identified with synthetic specimen of N-lactyltaurine by paper chromatography.

From the results obtained above, it was concluded that the constituents of two components with R_f 0.85 and 0.25 obtained from the starting glycolipide must be fatty acid combined with choline and *N*-lactyltaurine combined with trisaccharide respectively.

The starting glycolipide was not decomposed into the two components when it was treated with a weak acidic Amberlite IRC 50 or weak alkaline Amberlite IR 4B.

As mentioned above, it has been revealed that glycolipide obtained from oyster is decomposed by treatment with strong ionic exchanger into the two components, a cationic component consisting of choline and fatty acid, and an anionic component containing *N*-lactyltaurine and trisaccharide. The results show that these two components must be combined with an ionic linkage to form the mother glycolipide.

As for the structure of the cationic component, the fatty acid must exist in ester form with the carbinol radical of choline, because the only basic center of this component is that of quaternary ammonium nitrogen in choline portion.

On the other hand, the anionic component was decomposed by alkaline hydrolysis to give *N*-lactyltaurine and its structure should be represented as a possible combination of each one mole of *N*-lactyltaurine and trisaccharide.

The trisaccharide has been previously confirmed to be D-glucopyranosyl-$<$ 4.1 $>$-D-glucopyranosyl-$<$ 4.1 $>$-L-fucopyranose and its reducing group must take part in a glycosidic linkage, because before hydrolysis of the anionic component, all of its hydroxyl groups, except the reducing group, have been proved not to be substituted with other moieties (*2*). The possible radical in *N*-lactyltaurine to be substituted is only its hydroxyl group, so the reducing group of trisaccharide must be combined with hydroxyl group of *N*-lactyltaurine to form *O*-glycosidic linkage and acidic center of the anionic component should be the sulfonate group of taurine moiety.

As conclusion, the total structure (Scheme 1), *O*-(14-methyl-4-pentadecenoyl)-choline *N*-[*O*-(L-fucopyranosyl-$<$ 1.4 $>$-D-glucopyranosyl-$<$ 1.4 $>$-D-gluco-

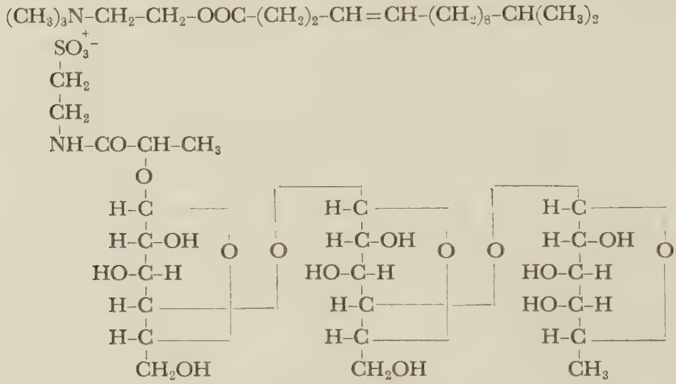
* A solvent system lutidine-collidine-H_2O (1 : 1 : 1) was used.

** A solvent system methyl cellosolve-NH_4OH-H_2O (80 : 5 : 15) was used.

pyranosyl)-lactyl]-taurate, could reasonably be proposed for the glycolipide isolated from oyster.

Scheme 1

Total structure of the glycolipide.



EXPERIMENTAL

Analysis of the glycolipide obtained from the soft tissues of oyster (Fig. 1-(J) reported in the first paper of this series (*1*)). Calcd. for $C_{41}H_{82}O_{21}N_2S$: C, 52.47; H, 8.20; N, 2.78; S, 3.18. Found: C, 51.84; H, 7.65; N, 2.39; S, 3.23.

Detection of Lactic Acid—500 mg. of the glycolipide was heated with 50 ml. of 10 per cent HCl in a boiling water-bath for 3 hours. After cooling, the separated fatty acid was extracted with petroleum ether and the aqueous layer obtained was extracted with ether repeatedly.

The ether extracts were combined and evaporated. The residue was examined by paper chromatography.

Solvent	R_f
Methyl cellosolve-NH_4OH-H_2O (80 : 5 : 15)	0.64
Butanol-formic acid-H_2O (10 : 2 : 15)	0.60

Each spot was identified with that of authentic lactic acid.
p-Bromophenacyl ester: m.p. 113°,

Analysis Calcd. for $C_{11}H_{11}O_4Br$: C, 45.99; H, 3.83.
Found : C, 45.38; H, 4.12.

Determination of Lactic Acid—The sample exactly weighed (10-20 mg.) was hydrolysed and after removal of fatty acid as above, lactic acid content was determined by Barker and Summerson's method (*5*) colorimetrically.

Detection of Nitrogenous Components—The aqueous layer obtained after removal of lactic acid by ether extraction was evaporated *in vacuo*. The residue was dissolved in 40 ml. of water and to the solution were added with stirring sodium acetate and a slight excess of warm solution saturated with ammonium reineckate. The mixture was kept at room temperature for one hour, the precipitate appeared was centrifuged, washed with water and dissolved in 50 ml. of acetone. To the acetone solution, a hot saturated solution of Ag_2SO_4 was added to decompose the reineckate. After removal of resulted precipitate by

centrifugation, excess of silver ion in supernatant was removed with H_2S. The silver-free solution was concentrated under reduced pressure and $BaCO_3$ was added to remove sulfuric acid. The excess of $BaCO_3$ and $BaSO_4$ occurred were filtered and the filtrate was further concentrated to dryness to yield 45 mg. of brown pasty residue. The residue was dissolved in 30 ml. of water and passed through a column of Amberlite IR 120 (H^+-type). The column, after washing with water, was eluted with 2 *N* HCl. The solvent was evaporated from the effluent to dryness to give 30 mg. of slightly yellow crystal.

The R_f values of crystal thus obtained were as follows:

Solvent	R_f
Lutidine-collidine-H_2O (1 : 1 : 1)	0.45
Butanol-acetic acid-H_2O (25 : 6 : 25)	0.28

Each spot was identified with that of authentic choline chloride.

Picrate: m.p. 239°,

Analysis. Calcd. for $C_5H_{14}ON \cdot C_6H_2O_7N_3$: C, 39.76; H, 4.82; N, 16.89.
Found : C, 39.21; H, 4.56; N, 16.40.

To the filtrate from choline reineckate was added a hot solution of Ag_2SO_4 to remove an excess of ammonium reineckate. The precipitate occurred was centrifuged, and the supernatant was treated with H_2S to remove silver ion. The silver-free solution was concentrated to about 30 ml. *in vacuo*. To this solution were added dropwise 10 per cent Na_2CO_3 and 25 per cent mercuric acetate keeping slightly alkaline reaction. The resulted precipitate was suspended in 100 ml. of water and decomposed with H_2S to remove mercury and filtered. The filtrate was evaporated to dryness and the residue was recrystallized from aqueous ethanol to give colorless needles. Yield 30 mg., *Analysis.* Calcd. for $C_2H_7NO_3S$: C, 19.20; H, 5.60; N, 11.20. Found: C, 19.26; H, 5.10; N, 10.96.

The R_f values of this crystal were as follows:

Solvent	R_f
Lutidine-collidine-H_2O (1 : 1 : 1)	0.43
Phenol-H_2O (5 : 1)	0.36
Butanol-acetic acid-H_2O (25 : 6 : 25)	0.16

Each spot was identified with that of taurine.

Determination of Choline—30–50 mg. of the glycolipide exactly weighed was heated with 20 ml. of 10 per cent HCl in a boiling water-bath for 2.5 hours. After cooling, the mixture was extracted with ether to remove fatty acid, and from the aqueous layer solvent and HCl were evaporated to dryness *in vacuo*. Choline content in this residue was determined by Glick's method (*6*).

Determination of Fatty Acid—The exactly weighed sample (10–15 mg.) was hydrolysed as above and fatty acid content was determined by Bloor's method (*7*).

Determination of Taurine—The exactly weighed sample (10–15 mg.) was hydrolysed and taurine was determined colorimetrically by ninhydrin method (*8*).

O-(14-Methyl-4-pentadecenoyl)-choline (*Cationic component*)—A solution of 30 mg. of the glycolipide in 10 ml. of water was passed through a column packed with 40 ml. of Amberlite IR 120 (H^+-type). The column was eluted with 80 per cent ethanolic 0.1 *N* HCl after washing with water. In order to remove both HCl and water avoiding further hydrolysis of the decomposition product, the effluent was neutralized by addition of Amberlite IR 4 B (OH^--type) and the filtrate obtained after removal of the resins was evaporated *in vacuo* to obtain a slightly yellow paste. Picrate: m.p. 95–6°.

Analysis Calcd. for $C_{21}H_{42}O_2N \cdot C_6H_2O_7N_3$: C, 57.02; H, 7.80; N, 9.85.
Found : C, 56.32; H, 8.12; N, 9.20.

Anionic Component—In above treatment, a solution of glycolipide which was passed through Amberlite IR 120 and removed from cationic component was combined with washings and passed through a column packed with 40 ml. of Amberlite IRA 410 (OH^--type). The column was eluted with 80 per cent ethanolic 0.1 *N* HCl after washing with water. The effluent was evaporated *in vacuo* below 30° to dryness avoiding further hydrolysis of the component and slightly yellow residue was obtained.

Synthesis of N-Lactyltaurine—To a suspension of 8.0 g. of finely powdered taurine in 50 ml. of dry pyridine was added 10 g of acetyllactic acid chloride (*9*) with stirring. After the mixture was allowed to stand at room temperature for 24 hours, it was filtered. The filtrate was evaporated *in vacuo* and the residual paste was dissolved in 15 ml. of ethanol. To the ethanolic solution a large excess of ethyl acetate was added; the syrupy precipitate occurred was separated from supernatant, dissolved in 50 ml. of 28 per cent NH_4OH and allowed to stand in room temperature for 20 hours. The solvent was evaporated *in vacuo* and residue was dissolved in 50 ml. of water. The aqueous solution was passed through a column of Amberlite IR 120 (H^+-type) and effluent obtained was passed through a column of Amberlite IR 4 B (OH^--type). The column was eluted with 1 per cent Na_2CO_3 and the alkaline effluent was passed through a column of IR 120 (H^+-type). Resulted effluent was evaporated *in vacuo* to obtain a pale yellow syrup. Yield 3.2 g. *S*-Benzylthiuronium salt: mp. 225–6°.

Analysis Calcd. for $C_5H_{10}O_5NS \cdot C_8H_{11}N_2S$: C, 42.94; H, 5.83; N, 11.56.
Found : C, 42.63; H, 5.95; N, 11.23.

Alkaline Hydrolysis of Anionic Component—8 mg. of the anionic component obtained by ion exchange treatment was heated with 10 ml. of 1 *N* NaOH for 2 hours in boiling water-bath. After cooling, the alkaline solution was successively treated with Amberlite IR 120 (H^+-type) and 4 B (OH^--type) as in the case of above synthesis. The acidic effluent obtained was concentrated *in vacuo* and examined by paper chromatography.

Solvent	R_f
Methyl cellosolve-NH_4OH-H_2O (80 : 5 : 15)	0.77
Phenol-H_2O-formic acid (75 : 24 : 1)	0.52

Each spot was identified with that of synthetic specimen of *N*-lactyltaurine.

SUMMARY

The structures of a trisaccharide and fatty acid obtained from a new glycolipide contained in the soft tissues of oyster were reported previously (*1–4*). The further investigations on the remaining portions of the glycolipide and the proposition of its total structure are given.

This glycolipide was decomposed by strong ionic exchanger into two components, a cationic components consisting of choline and fatty acid and an anionic component consisting of taurine, lactic acid and trisaccharide.

The total structure of this glycolipide was proposed as *O*-(14-methyl-4-pentadecenoyl)-choline *N*-[*O*-(L-fucopyranosyl-< 1.4 >-D-glucopyranosyl-< 1.4 >-D-glucopyranosyl)-lactyl]-taurate.

The author wishes to express his gratitude to Prof. S. Akiya of Tokyo Medical and Dental University, Prof. T. Ukita and Assistant Prof. S. Okui of the University of Tokyo for their kind advices and encouragements.

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STUDIES ON THIAMINOKINASE FROM BAKER'S YEAST*

II. NUCLEOTIDE SPECIFICITY

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In the previous paper of this series (*1*), the purification and properties of yeast thiaminokinase was described, together with observations on the rôle of divalent cations in this reaction. In the course of these studies, it was found that this enzyme has a broad nucleotide specificity and although at different rates, it catalyzes the transfer of pyrophosphoryl group to thiamine from GTP**, ITP, UTP, and CTP, besides ATP. Moreover, it was noted that under certain conditions ATP was completely ineffective as phosphate donor and GTP or ITP was very effective, while under other conditions ATP was much more effective than any other NTP. It seemed to be of interest that the nucleotide specificity of this enzyme depended remarkably on the experimental conditions under which the reaction was carried out.

At first some of the above observations were made with partially purified enzyme which contained some activity of other enzymes such as NDPkinase, adenylate kinase, and ATPase. Therefore, it was decided to purify the enzyme more extensively, in the hope of obtaining a more precise information on the relationship of the nucleotide specificity to experimental conditions.

With the enzyme isolated in a considerably purified state and freed of NDPkinase and adenylate kinase activity, above observations were reaffirmed with a more careful analysis of the factors involved. Furthermore, some evidences were obtained for the fact that a single enzyme catalyzes the transfer of pyrophosphate group from various NTP to thiamine. The relative effectiveness of these phosphate donors was found to be determined by such factors as might influence the formation of metal-NTP complex acting as substrate in this reaction.

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** The following abbreviations were used: NTP, nucleoside triphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; ITP, inosine triphosphate; ADP, adenosine diphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate; CDP, cytidine diphosphate; IDP, inosine diphosphate; TDP, thiamine diphosphate; NDPkinase, nucleoside diphosphokinase; Tris, tris(hydroxymethyl)aminomethane; and DEAE-cellulose, diethylaminoethyl-cellulose.

A preliminary report of this investigation has been published elsewhere (*2*). In this paper are presented the details of further studies concerning the effect of various factors on the nucleotide specificity. The reaction mechanism of the enzyme has also been presented elsewhere (*3*).

EXPERIMENTAL

Materials and methods were the same as those described in the previous paper (*1*). Thiaminokinase was prepared from baker's yeast by the same procedure as previously described (*1*). The preparation of highest purity obtained by column chromatography on DEAE-cellulose was used throughout the experiments unless otherwise specified, after being checked for the absence of NDPkinase activity. The pH measurements were made with a Tôyô Rika pH meter standardized with buffers at room temperature.

The assay method for thiaminokinase activity during the enzyme purification was the same as that described in the preceding report (*1*).

The reaction conditions will be referred in each case. 1.5 μ moles of neopyrithiamine was added to the TDP assay system to eliminate the residual thiaminokinase activity as a contaminant in the apocarboxylase preparation.

RESULTS

Effect of pH—As may be seen from the data given in Fig. 1, the pH-activity curve differed markedly with each NTP. ATP was the most active at alkaline pH values and the optimum was found to be around pH 8.6–8.8. A significant decrease in rate was observed on acid side. The pH optima for ITP and UTP were at 6.8 and 8.2, respectively. When GTP was used as phosphate donor, the reaction had a broad pH optimum ranging from 6 to 8. In these experiments the Mn concentration was fixed to 4×10^{-4} *M*, and other conditions are given in the legend to Fig. 1.

These results should not be interpreted only in terms of pH, because as will be described later, it was also noted that these pH optima depended on the relative concentration of NTP and activator cations.

Effect of Mn Concentration—A comparison of relative activity of each nucleotide as a function of Mn concentration is given in Fig. 2. In this experiment pH of the reaction medium was selected at 8.4 where all the phosphate groups in NTP are known to be in dissociated form. Under these conditions the nucleotide specificity was found to be quite dependent on Mn concentration; the optimal Mn concentration for GTP was in the neiborhood of 1×10^{-5} *M*, where ATP and CTP were practially inactive. At such a high concentration of Mn as was required for maximal activity of ATP, very little activity of GTP was observed. Thus, each nucleotide had its own Mn optimum, and an excess of Mn resulted in an appreciable inhibition of the rate.

The values in Fig. 2. are expressed in terms of relative specific activity, and the activity ratio of each nucleotide at optimal pMn was A : U : G : I : C = 1 : 1 : 0.55 : 0.55 : 0.25. Other conditions are indicated in the legend to Fig. 2.

In view of the above results, the effect of pH on enzyme activity was reexamined at optimal concentration of Mn with each nucleotide. As shown

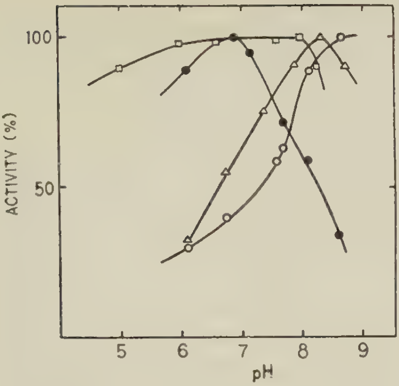

FIG. 1. Activity at fixed metal concentration as a function of pH.

The reaction mixture composed of 30 μ moles of Tris-maleate buffer, pH as indicated; 0.6 μ mole of $MnSO_4$; 0.15 μ mole of thiamine; 0.15 μ mole of NTP; and the enzyme to a final volume of 1.5 ml. After incubation for 60 minutes at 30° the reaction was terminated by heating in a boiling water bath for 2 minutes. A suitable aliquot was taken and assayed for TDP formed (*1*). The values are expressed as per cent of optimal activity with each nucleotide. —○— ATP, —△— UTP, —□— GTP, —●— ITP.

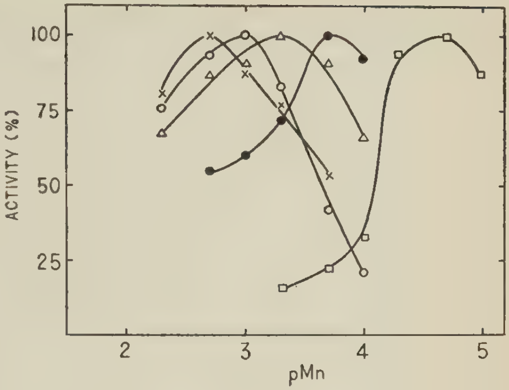

FIG. 2. Dependence of nucleotide specificity on Mn concentration.

The reaction mixture contained; 30 μ moles of Tris-maleate buffer, pH 8.4; $MnSO_4$, as specified; 0.15 μ mole of thiamine; 0.15 μ mole of NTP; and the enzyme to a final volume of 1.5 ml. Other conditions were the same as described in Fig. 1. The values are expressed as per cent of optimal activity with each nucleotide. —×— CTP, —○— ATP, —△— UTP, —●— ITP, —□— GTP.

in Fig. 3, the pH optimum for all NTP was found to be above 8.0. The pH-activity curves in Fig. 1 were obtained at a fixed Mn concentration which was far higher than that required for maximal activity of GTP or ITP. From Figs. 1 and 3, it might be deduced that when a concentration of Mn in excess of the optimum is employed, the pH optimum would shift toward lower values. Therefore, the differences observed in pH optima with various NTP might be the result of different affinity of Mn to each NTP.

Effect of Mg—In the above experiments, Mn was used as an activator cation. When it was replaced by Mg, comparative effectiveness of various NTP became entirely different from that obtained with Mn. As illustrated in Table I, GTP was the most active, and ITP and UTP showed smaller activity in the order named. On the other hand, little activity was observed when ATP or CTP was used as phosphate donor. Probably low concentrations of ATP or CTP could not form the complex which acts as a true substrate in this reaction, because these nucleotides bind Mg less storongly than does GTP or ITP under such conditions. In order to save expensive substrates, these experiments were performed at the NTP concentration of 1×10^{-4} *M*, where the saturation of the enzyme with ATP was not reached (*1*). When ATP was used at a higher concentration of 1×10^{-3} *M*, a significant activity of ATP was observed even when Mg was used as an activator cation.

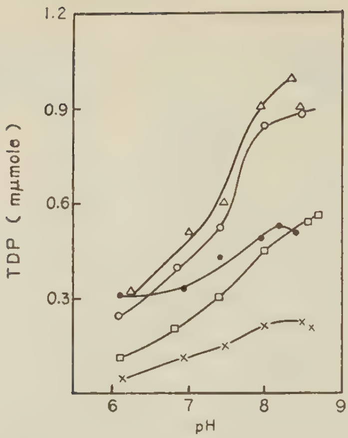

FIG. 3. pH dependence of thiaminokinase reaction at optimal Mn concentration for each NTP.

The reaction conditions were the same as those described in Fig. 1 except that the Mn concentration were 2×10^{-3} *M*, 5×10^{-4} *M*, 2×10^{-4} *M*, 2×10^{-5} *M* and 2×10^{-3} *M*, for ATP, UTP, ITP, GTP and CTP, respectively. The values are expressed as mμ moles of TDP formed per hour. —○— ATP, —△— UTP, —●— ITP, —□— GTP, —×— CTP.

TABLE I

Comparative Effect of Various NTP as Phosphate Donor with Mg as Activator Cation

Additions	TDP formed
	(*mμmoles per hour*)
ATP	0.24
CTP	0.14
UTP	0.74
ITP	1.40
GTP	1.98

The composition of the reaction mixture was as follows: Tris-buffer, pH 8.2, 0.02 *M*; $MgSO_4$, 0.01 *M*; thiamine, 1×10^{-4} *M*; NTP, 1×10^{-4} *M*; and thiaminokinase to a final volume of 1.5 ml. Other conditions were the same as those given in Fig. 1.

Effect of Citrate Buffer—In a series of the experiments which were carried out in citrate buffer, pH 6.0, GTP was the most active phosphate donor, as shown in Table II. At the nucleotide concentration of 1×10^{-4} *M*, ITP was nearly as effective as GTP, and UTP was about 30 per cent as active as GTP, while ATP and CTP were again almost ineffective. These results are very similar to those obtained with Mg and Tris buffer in the presence of 1×10^{-4} *M* NTP (Table I). An explanation for these results is that the effective metal

concentration would be very much reduced in the citrate buffer because of the strong chelating effect of the citrate anion. Therefore, the relative effectiveness of various NTP becomes similar to that observed with low Mn con-

TABLE II

Effect of Various NTP on Thiaminokinase Reaction in Citrate Buffer

Additions	TDP formed	
	Nucleotide 1×10^{-5} *M*	Nucleotide 1×10^{-4} *M*
	(*mμmoles*)	(*mμmoles*)
ATP	0	1.1
CTP	0	0
UTP	0	4.7
ITP	0.6	10.8
GTP	5.6	14.4

The composition of the reaction mixture was as follows: Citrate buffer, pH 6.0, 1.7×10^{-2} *M*; $MnCl_2$, 2×10^{-3} *M*; $MgSO_4$ 3.3×10^{-3} M; thiamine, 1×10^{-4} *M*; NTP, as specified; and thiaminokinase (after ammonium sulfate fractionation) to a final volume of 3.0 ml. Other conditions were the same as those described in Fig. 1.

centrations (*cf.* Fig. 2) and that observed with Mg (Table I). ATP has a small but significant activity at 1×10^{-4} *M*, and in this case, the higher concentration of ATP (1×10^{-3} *M*) was not effective.

Evidence for the Identity of the Enzyme—From the foregoing results, the nucleotide specificity seemed to be quite dependent upon experimental conditions, but there remained two possibilities; First, that these activities of various NTP are due to contamination of different enzymes providing the mechanism whereby these NTP would be converted to one particular active nucleotide, which would be the specific substrate in this reaction. In this case a small amount of the latter should also be present as a contaminant in enzyme or nucleotide preparations. The second possibility is that these activities are due to the presence of separate enzymes, each of which is specific for one particular nucleotide.

In view of the fact that ATP was the most effective of all the NTP under certain conditions, whereas it became less active than other NTP under other conditions, the first possibility seemed to be improbable. Moreover, NDPkinase activity was not detected in the purified enzyme. In order to exclude the second possibility, chromatographic distribution of the enzyme activity was followed with ATP and ITP as phosphate donor. Activity with these phosphate donors was always recovered in the same fraction and with almost a constant ratio during the anion exchange chromatography on DEAE-cellulose column. (Fig. 4).

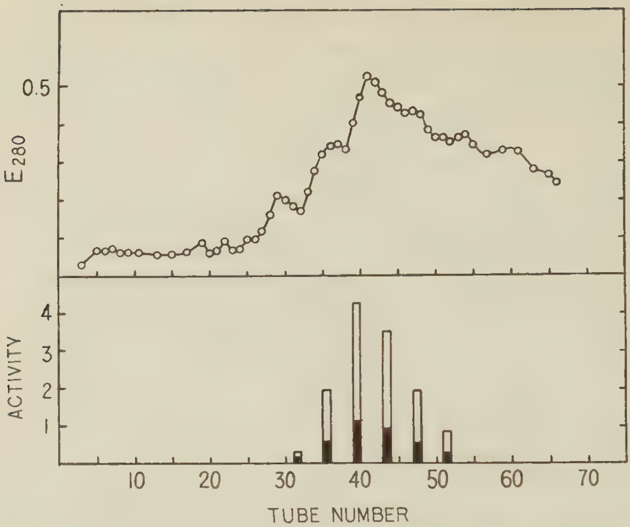

FIG. 4. Identity of ATP-thiamine pyrophosphokinase with ITP-thiamine pyrophosphokinase.

About 55 mg. of the enzyme was adsorbed on a column (1.1×10 cm.) of DEAE-cellulose which had been equilibrated with 0.05 *M* Tris buffer, pH 8.6. The chromatogram was developed by gradient elution based on the Tris buffer-NaCl system, with 200 ml. of 0.05 *M* Tris buffer, pH 8.6, in a mixing vessel, and with 0.4 *M* NaCl in 0.05 *M* Tris buffer, pH 8.6, in the reservoir vessel. The eluate was collected at a rate of 15 ml. per hour in 5-ml. fractions. Each fraction was assayed for protein (E_{280}, continuous curve) and enzyme activity with ATP (upper stepped curve) and ITP (shaded stepped curve) as phosphate donor. The enzyme activity was expressed as mμ mole $\times 10^{-1}$ of TDP formed per ml. The assay conditions were the same as described in the previous paper (*1*) except that 0.3 μ mole of $MnSO_4$ was added as an activator when ITP was the phosphate donor.

Further, if these activities are catalyzed by separate enzymes, each of which is specific for one particular substrate, an additive effect would be expected by further addition of the second NTP to the enzyme preparations which has already been saturated with one NTP. However, as may be seen from Table III, the results did not indicate an additive effect, and rather a marked inhibition was observed in some cases. In these experiments, 0.15 μ mole of each NTP was added to the enzyme preparation which was saturated with 1×10^{-3} *M* ATP. As the concentration of the second NTP was one-tenth that of ATP, the decrease in metal concentration would not be the cause of the inhibition. The lower part in Table III represents the activity of each NTP alone at 1×10^{-4} *M* concentration under these conditions. The activity of ITP and GTP was very small because Mn was present in greater excess of optimal concentrations required for maximal activity of these NTP.

Effect of Substrate Concentration—From the experiment shown in Table III, GTP seems to have a stronger affinity to the enzyme than ITP, the former

TABLE III

Effect of Various NTP on Thiaminokinase Reaction

Additions	TDP formed
	(*mμmoles per hour*)
ATP	3.25
ATP+CTP	2.92
ATP+GTP	0.70
ATP+UTP	3.21
ATP+ITP	1.63
CTP	0.59
GTP	0.11
UTP	1.45
ITP	0.45

The reaction mixture contained : 30 μ moles of Tris buffer, pH 8.6 ; 3 μ moles of $MnSO_4$; 0.15 μ mole of thiamine : 1.5 μ moles of ATP and/or 0.15 μ mole of other NTP ; and the enzyme to a final volume of 1.5 ml. Other conditions were the same as those given in Fig. 1.

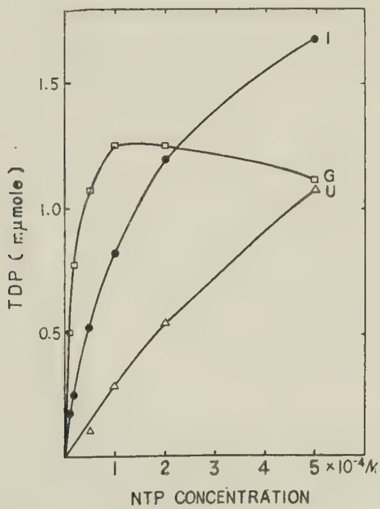

FIG. 5. Effect of nucleotide concentration on thiaminokinase reaction.

The reaction mixture was composed of 30 μ moles of Tris-maleate buffer, pH 8.4 ; 60 μ moles of $MgSO_4$; 0.15 μ mole of thiamine ; GTP, ITP or UTP, as specified ; and thiaminokinase to a final volume of 1.5 ml. Other conditions were the same as described in Fig. 1.

—●— ITP, —□— GTP, —△— UTP.

being more inhibitory than the latter when added with ATP, and low concentrations of GTP are much more effective than ITP (Table II). As it was difficult to determine K_m value accurately with each NTP under the same conditions, saturation curves with GTP, ITP, and UTP were studied. In this experiment, 4×10^{-2} M Mg was used as an activator to avoid the inhibitory effect caused by excess of Mn ions.

As is clear from Fig. 5, GTP saturated the enzyme at lowest concentrations and, therefore, when compared at the nucleotide concentration of 2×10^{-4} M or below, GTP was more effective than either ITP or UTP, whereas at the concentration of 5×10^{-4} M, ITP became more active than GTP. Saturation curves for ATP and CTP could not be obtained under the same conditions because they showed very little activity when Mg was used as activator. In the preceding report, K_m for ATP was calculated as 1.0×10^{-4} M in the presence of Mn (*1*).

DISCUSSION

From the results reported here, it seems reasonable to assume that one and the same enzyme is responsible for the reaction and each NTP would react as such, possibly for reasons of the broad nucleotide specificity of this enzyme.

In the preceding report of this series (*1*), it was pointed out that the enzyme activity with ATP as phosphate donor is affected by the species and concentrations of the activator cations, and pH of the reaction medium. Consequently, metal-ATP complex was postulated to be a possible substrate form for the reaction, as it has already been assumed in several cases of other phosphorylating enzymes by previous workers (*4–8*). In the present study the influence of these factors on nucleotide specificity was examined and found to be also very striking. In this case, therefore, the metal-NTP complex rather than free NTP would be again considered as the true substrate for the reaction and its dissociation constant might differ with each nucleotide. The species and concentrations of metal would be of primary importance to the formation of this complex, the formation constant of which would be affected by pH of the reaction medium.

The strong inhibition of the rate observed at higher levels of Mn ions is to be explained. Probably, a specific type of Mn-NTP complex is required as phosphate donor for the reaction. At a higher concentration of Mn, another type of complex would be formed between metal and NTP which would be inactive and sometimes competitively inhibitory. The alkaline pH would favor the formation of the latter complex because of the increase in dissociation of the phosphate group in NTP molecule. Although an excess of Mn was not inhibitory at low pH, it depressed the reaction rate above certain pH which was determined by the relative concentration of Mn to NTP. Therefore, the apparent pH optimum shifts toward the acid side with increasing concentration of Mn.

Walaas (*9*) has recently determined the formation constant of various mononucleotide complexes with Ca, Mg, Co, and Mn, by means of a physicochemical method. From his data, no such striking difference in the formation

constants as might be expected from observations in the present study seems to exist among various NTP. This discrepancy might be attributed to some extent to active participation of enzyme. At present, it is not clear whether the above results are due to the difference in the dissociation constant of each metal-NTP complex or to that in the affinity of the complex to the enzyme surface.

It has been known that some enzymes are not absolutely specific for one particular nucleotide, various other nucleotides being more or less active with the same enzyme preparation. In most of these cases, however, the effectiveness of nucleotides was compared only under the optimal conditions for the most effective nucleotide, and usually for adenine nucleotide. Only with ATPase, some papers (*10–12*) have recently appeared which treat the relative effectivenss of various NTP under different conditions.

From the data shown in Fig. 2. it may be said that at lower concentrations of Mn, the nucleotides having hydroxyl group in 6-position on pyrimidine ring are more active, and the enzyme seems to be specific for GTP, ITP, and UTP. On the other hand, at higher concentrations of Mn, this group of nucleotides become less active than that having 6-amino group on the pyrimidine ring.

These results seem highly significant, especially in view of the fact that in most of the reactions which have been reported to date to require guanosine nucleotide, inosine nucleotide was also active as phosphate donor or acceptor (*13*, *14*). In IDPase reaction (*15*), it was reported that the enzyme catalyzes the dephosphorylation of IDP, GDP and UDP, but is inactive on ADP and CDP. These observations might be explained by a close resembrance of GTP and ITP in their behavior towards metal ions.

It is not intended to extend these results as a general rule to all the reactions involving NTP. Obviously, there must also be some enzymes which are strictly specific for one particular nucleotide, and the above results might be due to the reaction mechanism of this enzyme which involves a specific type of transphosphorylation, *i.e.* the transfer of pyrophosphoryl group. However, the present results certainly stress the necessity of great care in the interpretation of the experiments of nucleotide specificity.

SUMMARY

1. With yeast thiaminokinase, some evidences for the dependence of nucleotide specificity on experimental conditions were presented.

2. The effect of Mn concentration was most striking; the optimal Mn concentration required for maximal activity of ATP was about 100 times greater than that required for that of GTP. Each nucleotide has its optimal pMn and in either side the reaction rate was depressed rather sharply.

3. In the presence of Mg, GTP and ITP were active as phosphate donor, whereas ATP and CTP were almost inactive.

4. When the reaction was carried out in citrate buffer, pH 6.0, relative effectiveness of various NTP was similar to that observed at low concentrations of Mn, or with Mg as an activator cation instead of Mn.

5. The possible reasons for the dependence of nucleotide specificity upon these factors were discussed.

6. The K_m values for each nucleotide could not be obtained under the same conditions, however, under certain conditions, GTP was found to have a higher affinity to the enzyme than ITP or UTP.

The author wishes to express his heartful gratitude to Prof. N. Shimazono for his continuous encouragements and interest during the course of this work. Thanks are also due to Prof. F. Dickens for his valuable suggestion at the Symposium on Water-Soluble Vitamins (*16*).

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GLUTAMIC ACID FORMATION FROM GLUCOSE BY BACTERIA

II. GLUTAMIC ACID AND α-KETOGLUTARIC ACID FORMATION BY BREVIBACTERIUM FLAVUM, No. 2247.

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In our previous report, evidence was obtained for the presence of hexokinase, phosphohexose isomerase, phosphohexokinase, aldolase, DPN*-linked glyceraldehyde-3-phosphate dehydrogenase, TPN-linked glucose-6-phosphate dehydrogenase, aconitase, TPN-linked isocitric dehydrogenase, succinic dehydrogenase, fumarase, TPN-linked malic enzyme (or malic dehydrogenase), oxalacetic carboxylase, and isocitritase in the cell-free extracts of a strain of *Brevibacterium flavum*, No. 2247, which grew on glucose as the sole source of carbon and accumulated a large amount of L-glutamate in its growing culture medium (*1*).

The present paper reports some results on the formation and metabolism of glutamic acid and α-ketoglutaric acid by an intact cell suspension as well as cell-free extract of this bacterium.

METHODS

Determination of Glucose by Glucose Oxidase—Glucose was determined by the slightly modified manometric method of Keilin and Hartree (*2*), using "Deoxin" (Nagase-sangyo Co.), a glucose oxidase preparation, which contained catalase. The main compartment of the manometric flask was filled with 1 ml. of 0.2 *M* phosphate buffer pH 5.8, 1 ml. of the unknown solution (*ca.* pH 5.8), the sidearm with 0.5 ml. of "Deoxin" solution (8 mg. per ml.), and the center well with 0.2 ml. of 20 per cent KOH. Then "Deoxin" solution was tipped in after equilibration and the determination of oxygen uptake was carried out at 37°.

Determination of Urea by Urease—The method used was essentially that of Krebs and Henseleit (*3, 4*) and depended on the manometric determination of CO_2 formed from urea at pH 5.0.

Determination of α-Keto Acids—α-Ketoglutaric acid and pyruvic acid were determined as their 2,4-dinitrophenylhydrazones by the slightly modified colorimetric method of Friedemann and Haugen (*5*) at 420 and 540 mμ.

* The abbreviations used throughout this paper include TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; αKG, α-ketoglutaric acid; DiNPH, 2,4-dinitrophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane; ΔE_{340}, increment of optical density at 340 mμ.

Determination of L-*Glutamic Acid by* L-*Glutamic Decarboxylase*—L-Glutamic acid was determined by the manometric method (*6*) using acetone dried cells of *E. coli* "Crookes" strain, a specific L-glutamic decarboxylase preparation.

Test for Glutamic Dehydrogenase Activity—The oxidation of glutamate was measured by the increase of optical density at 340 mμ caused by the reduction of TPN or DPN.

Test for Transaminase Activity—L-Glutamic acid formed from L-amino acid and α-ketoglutaric acid was determined manometrically.

Ammonia was determined by the colorimetric method using Nessler's reagent. Oxygen uptake and CO_2 evolution were measured by the usual manometric methods. The methods for the preparation of resting cell suspension and of cell-free extract, paper chromatography of amino acids and keto acids, and determination of nitrogen content of cell materials were described in our previous papers (*1*, *7*).

RESULTS

Glutamic Acid Accumulation in the Growing Culture Medium—When *Brev. flavum*-2247 was aerobically growing in the medium described in the previous paper (*1*), L-glutamic acid was accumulated with decrease of glucose and urea, and the amount of glutamate formed did not decrease for 35 hours after the maximum accumulation as shown in Fig. 1. The fact that main part of glutamate accumulated was formed after the maximum growth and that

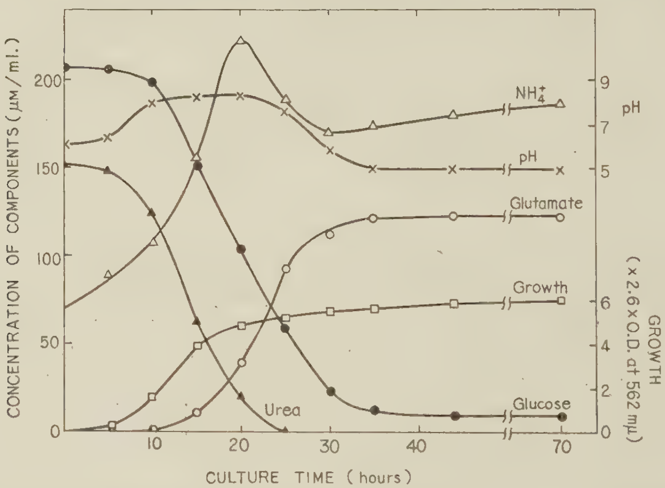

FIG. 1. Accumulation of glutamic acid in culture medium of *Brevibacterium flavum*, No. 2247.

Medium: glucose 3.6 per cent, urea 1 per cent, KH_2PO_4 1 per cent, $MgSO_4 \cdot 7aq.$ 0.04 per cent, Fe^{++} and Mn^{++} 2 p.p.m. each, thiamine 100 μg./liter, biotin 2 μg./liter, Ajinomoto's "Mieki" 0.1 per cent.

Condition: shaken at 30°.

urea was decomposed rapidly to ammonia, suggested a possibility that resting cells might also form glutamic acid from glucose and ammonia in a similar manner.

Aerobic Metabolism of Glucose by Resting Cells—Complete oxidation of one mole of glucose would proceed as follows:

$$C_6H_{12}O_6+6\,O_2=6\,CO_2+6H_2O$$

With this bacterium, oxygen absorption stopped at a point of four moles per one mole of glucose as shown in Fig. 2, and the evolution of 3.5 moles of

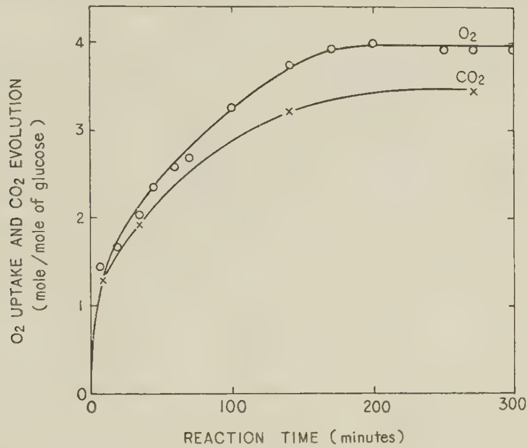

FIG. 2. Oxygen uptake and carbon dioxide evolution in the oxidation of glucose by resting cells. Reaction mixture contained 1 μM of glucose and cell suspension (1.1 mg. N) in a final volume of 2.5 ml. of 0.04 M phosphate buffer pH 7.0, 37°.

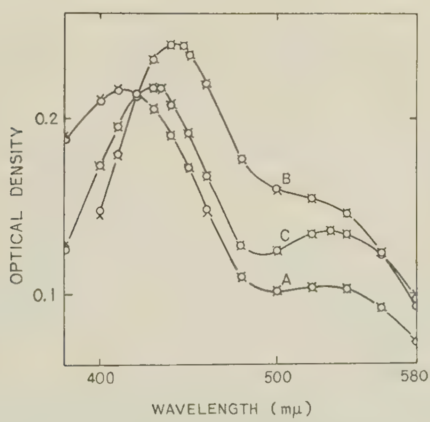

FIG. 3. Ultraviolet absorption spectra of 2,4-dinitrophenylhydrazones of standard α-ketoglutaric acid (A), pyruvic acid (B), and α-ketoisovaleric acid (transamination product from L-valine and αKG) (C) (—○—), and of reaction products (—×—) in 1 N sodium hydroxide.

CO_2 and the formation of 0.5 moles of α-ketoglutarate were observed. Paper-

chromatographic identification of αKG as an end product was further confirmed by the characteristic absorption spectrum (Fig. 3) and melting point of its DiNPH, which was recrystallized from water:

	melting point (uncorrected)
Standard αKG-DiNPH	210.9°
End product-DiNPH	210.9°
Mixture of above two specimens	210.9°

These results would be summarized in the following equation:

$$C_6H_{12}O_6+4\ O_2=3.5\ CO_2+0.5\ C_5H_6O_5+4.5\ H_2O$$

From a stoichiometric point of view, the other products would be negligible:

Formation of α-Ketoglutaric Acid and Glutamic Acid by Resting cells—As shown in Figs. 4 and 5, a large amount of αKG (in the absence of ammonium ions) or glutamate (in the presence of ammoniam ions) was formed aerobically

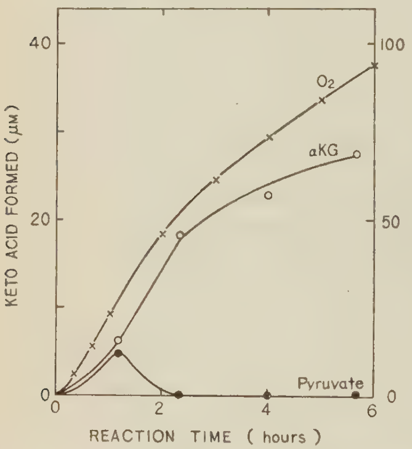

FIG. 4. Formation of α-ketoglutaric acid in the oxidation of glucose by resting cells.

Reaction mixture contained 50 μM of glucose and cell suspension (0.88 mg. N) in a final volume of 2.5 ml. of 0.1 *M* phosphate buffer pH 7.0. Shaken at 37°.

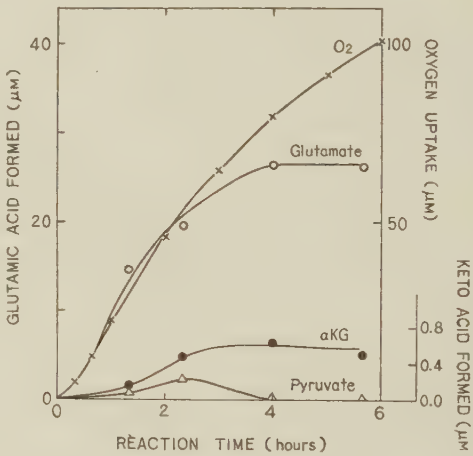

FIG. 5. Formation of glutamic acid in the oxidation of glucose by resting cells.

Reaction mixture contained 50 μM of glucose, 75 μM of ammonium chloride, and cell suspension (0.88 mg. N) in a final volume of 2.5 ml. of 0.1 *M* phosphate buffer pH 7.0. Shaken at 37°.

from glucose by a resting cell suspension. The other products detected by paper chromatography were: alanine, valine, pyruvic acid, αKG (in the presence of NH_4^+); pyruvic acid, and an unknown keto acid (in the absence of NH_4^+).

The latter unknown keto acid seemed to be α-ketoisovaleric acid, since its DiNPH showed the same paperchromatographic behaviors and the same absorption spectrum as that of the transamination product from valine and αKG (Fig. 3).

Metabolism of α-Ketoglutaric Acid and Glutamic Acid by Resting Cells—As

shown in Table I, neither glutamic acid nor αKG was decomposed by a resting cell suspension under the aerobic condition given in the table, where about 10 μM of glutamic acid and αKG could be formed, respectively, from 20 μM of glucose by the same amount of cells in one ml. of reaction mixture. However, after 5 hours very slight oxygen uptake and formation of 0.03 μM per ml. of αKG from glutamate were detected under the same condition.

Table I

Metabolism of α-Ketoglutaric Acid and Glutamic Acid by Resting Cells and Effect of Various Compounds

Substrate	Addition (μM/ml.)	Concentration of substrate (μM/ml.) after 0,	100,	150,	200,	300 min.
αKG	—	8.7	8.5	—	8.4	8.2
,,	— cells	8.7	—	—	—	8.2
Glutamate	—	10.0	10.2	10.1	10.1	10.0
,,	Glucose, 10	10.0	—	10.1	—	10.0
,,	Succinate, 20	10.0	—	10.1	—	9.6
,,	Acetate, 30	10.0	—	9.5_5	—	9.1
,,	Pyruvate, 20	10.0	—	9.4	—	8.7
,,	Oxalacetate 20	10.0	—	7.1	—	6.3_5

Reaction mixture contained reactants as given and cell suspension (0.35 mg. N) in a final volume of 1 ml. of 0.1 *M* phosphate buffer pH 7.0. Shaken under air at 37°.

Table II

Demonstration of Transamination Reaction by Resting Cells

Substrate (μM/ml.)	Glutamic acid changed (μM/ml.)	Product detected
Glutamate (10)+pyruvate (20)	−0.3	Alanine, αKG
Glutamate (10)+oxalacetate (20)	−3.9	Aspartate, αKG, pyruvate
Glutamate (10)	0.0	—
αKG (50)+L-aspartate (50)	+5.3	Glutamate, pyruvate
αKG (50)+DL-alanine (50)	+0.9	Glutamate, pyruvate
αKG (50)+NH_4Cl (50)	+0.5	Glutamate

Reaction mixture contained substrates and cell suspension (0.35 mg. N) in a final volume of ml. of 0.1 *M* phosphate pH 7.0. Incubated in a Thunberg tube for 150 minutes at 37° after evacuation.

That slight oxygen uptake was not affected by the addition of methylene blue, an oxido-reduction dye. As given in Table I, a marked decrease of glutamate could be observed, however, in the presence of various compounds, especially oxalacetate and pyruvate, and, in fact, transaminase activities were demonstrated in resting cells (Table II). These results showed also that

glutamic acid and α-ketoglutaric acid were permeable enough to be attacked by the intracellular enzymes of the cells.

Enzymes of Glutamic Acid Metabolism, and Formation of Glutamic Acid by Cell-free Extract—Cell-free extract, which was obtained by the sonic disintegration of washed cells as described in our previous paper (*1*), showed transaminase activities between αKG and aspartate, alanine, and valine, respectively, and also glutamic dehydrogenase activity, for which TPN was required as a coenzyme (Table III, Fig. 6, 7). Since this extract contained also aconitase and TPN-isocitric dehydrogenase (*1*), glutamic acid formation from citrate and ammonium ions by this extract would be expected, which was now proved as shown in Fig. 8, and the requirement of manganese ions and of catalytic amount of TPN for full activity suggested that isocitric and glutamic dehydrogenases were involved in this reaction. These coupled reactions carried out through the reduction and oxidation of coenzyme, TPN, were illustrated in Fig. 9.

FIG. 6. Formation of glutamic acid from α-ketoglutaric acid and L-amino acid (aspartic acid, —×—; alanine, —△—; valine, —○—) by cell-free extract. Reaction mixture contained 30 μM of sodium α-ketoglutarate, 50 μM of L-amino acid as indicated and cell-free extract (0.145 mg. N) in a final volume of 1 ml. of 0.1 *M* phosphate buffer pH 7.0, 37°.

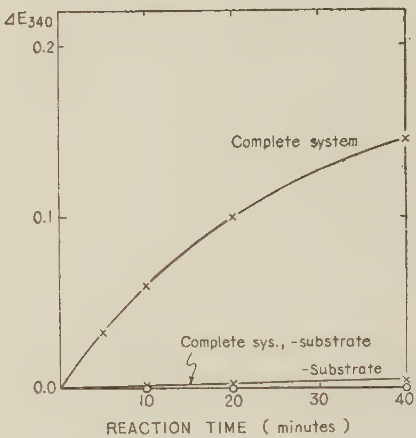

FIG. 7. L-Glutamic dehydrogenase activity of cell-free extract and its coenzyme specificity.

Complete system of reaction contained 10 μM of sodium L-glutamate, 0.25 μM of TPN (—×—) or DPN (—○—), and cell-free extract (0.155 mg. N) in a final volume of 1 ml. of 0.05 *M* Tris buffer pH 7.5, 23°.

TABLE III

Transaminase Activity of Cell-free Extract

System	L-Glutamate formed (μM/*ml.*)		
	L-Aspartate	L-Alanine	L-Valine
L-Amino acid+αKG	16.8	2.57	2.84
L-Amino acid	0.96	0.75	0.80
αKG+NH_4Cl	0.80	0.80	0.80
None	0.91	0.91	0.91

Reaction mixture contained 50 μM of L-amino acid, 30 μM of α-ketoglutarate, and cell-free extract (0.145 mg. N) in a final volume of 1 ml. 0.1 *M* phosphate buffer pH 7.0. Incubated for 4 hours at 37°.

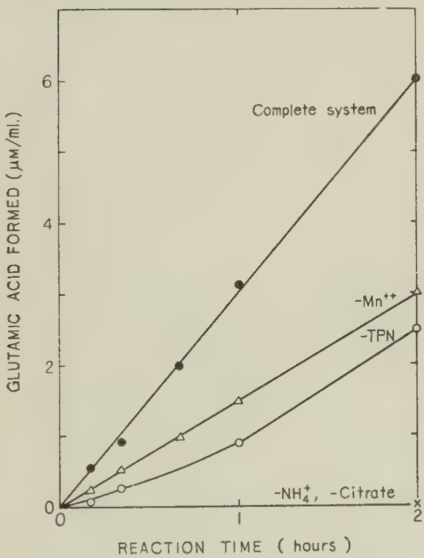

FIG. 8. Formation of glutamic acid from citrate and ammonium ions by cell-free extract.

Complele system of reaction contained 20 μM of sodium citrate, 30 μM of ammonium chloride, 0.5 μM of $MnSO_4$, 0.063 μM of TPN, and cell-free extract (0.195 mg. N) in a final volume of 1 ml. of 0.05 *M* Tris buffer pH 7.5, 30°.

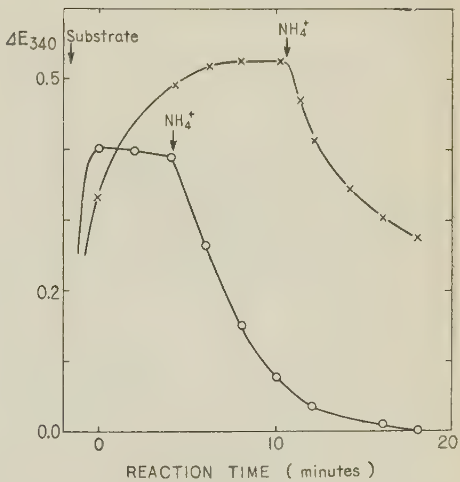

FIG. 9. Reduction of TPN by tricarboxylic acids and its reoxidation by the addition of ammonium ions in the presence of cell extract. Reaction mixture contained 0.063 μM of TPN, 0.5 μM of $MnSO_4$, cell-free extract (0.145 mg. N), and 1 drop of 0.02 *M* citrate (—×—) or isocitrate (—○—) in a final volume of 1 ml. of 0.05 *M* Tris buffer pH 7.5, 24°. One drop of 0.3 *M* ammonium chloride was added at the times indicated with arrows.

DISCUSSION

In the oxidation reaction of one mole of glucose, only α-ketoglutarate and carbon dioxide were the end products and the amount of αKG formed varied from 0.5 to 0.75 moles with various cell suspensions and under various conditions of the reaction. Therefore, the oxidative decomposition of glucose by this bacterium would occur not through a single pathway but through two pathways, that is, one pathway for αKG formation and another pathway for the complete oxidation, one of which may be the Krebs cycle, since most of enzymes of the Krebs cycle were detected in the cell extracts of this cells (*1*).

By intact cells, α-ketoglutarate and glutamate were not oxidized nor decarboxylated, but since transamination took place the inability of cells for the oxidation of αKG and glutamate can not be ascribed to an impermeability of these substances through the cell membrane. These results consist with the accumulation of αKG or glutamate as the end product in the oxidation reaction of glucose and suggest that the pathway of the complete oxidation of glucose would not contain αKG as an intermediate product, and therefore not belonging to the Krebs cycle, may be, for example, the glyoxylate cycle (*8*).

Existence of the glyoxylate cycle in this bacterium was suggested by the facts that isocitritase as well as various enzymes of the Krebs cycle were detected in it (*1*) and that αKG was accumulated when acetate was oxidized by an intact cell suspension*.

Glutamic dehydrogenase activity of a cell extract was considerably high, although the oxidation of glutamate by intact cells was negligible. Similar results had been obtained in the case of *Micrococcus glutamicus* by Kinoshita *et al.* (*9*). They postulated (*10*, *11*) from the positive effect of methylene blue for oxygen uptake in a system of glutamate (or citrate) and a cell-free extract that the inability of their intact cells for the oxidation of glutamate despite the presence of strong glutamic dehydrogenase might be due to the difficult reoxidation of reduced TPN by oxygen through the terminal respiratory enzyme systems. For this point however, further investigation would be required since cell-free extracts of bacteria can not be generally expected to contain these terminal respiratory enzyme systems because of possible degeneration during the preparation and the insoluble properties of these enzyme systems (*12*).

The data of the present experiment with the cell extract demonstrated two possible enzymic reactions for the glutamic acid formation from α-KG, namely transamination and reductive amination, each of which proceeded rapidly enough to account for the glutamate formation by intact cells.

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* unpublished data.

SUMMARY

1. α-Ketoglutarate and, when ammonium ions were present, glutamate were formed aerobically from glucose by resting cells of *Brevibacterium flavum*, No. 2247.

2. Glucose was not completely oxidized by resting cells, and only α-ketoglutarate and CO_2 were the end products, for instance,

$$C_6H_{12}O_6+4\ O_2=3.5\ CO_2+0.5\ C_5H_6O_5+4.5\ H_2O$$

3. α-Ketoglutarate and glutamate were not oxidized nor decarboxylated but transaminated by intact cells.

4. Cell-free extract showed TPN-linked glutamic dehydrogenase activity as well as transamination between α-ketoglutarate and aspartate, alanine, and valine, respectively.

5. Rate of the glutamate formation by cell-free extract either from α-ketoglutarate and aspartate or from citrate and ammonium salt could account for the glutamate formation by resting cells.

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STUDIES ON PHOSPHOLIPIDE METABOLISM

IX. A COLUMN CHROMATOGRAPHIC SEPARATION OF PHOSPHOLIPIDES IN LIVER AND HEART*

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An available method to fractionate the individual component in the mixed phospholipides from the tissues has been desired in the studies on chemistry and metabolism of phospholipides. However, the separation and purification of the individual phospholipide have been a difficult problem for a long time. Recently, column chromatographic technique, which was carried out with various adsorbents, has been recognized as an effective method to fractionate the phospholipides.

Taurog *et al.* (*1*) were able to separate the phospholipides into two fractions, the choline-containing and the non-choline-containing phospholipides, by adsorption chromatography on magnesium oxide. Moreover, a method was reported by Hanahan *et al.* (*2*). in which aluminium oxide was used as the adsorbent and lecithin was prepared in good yield and in highly purified form. None of these procedures, however, has permitted the subsequent isolation of the other phospholipides. Using silicic acid and celite, McKibbin was able to separate a polyglycerophosphatide (*3*) and an inositide (*4*) from liver. Since this time, the column chromatography on silicic acid has been applied to the separation of the phospholipides. Lea *et al.* (*5*) applied the silicic acid to the separation of the two major components of egg-yolk phospholipides. Hanahan *et al.* (*6*) presented a procedure, by which the various components in the mixed phospholipides from livers and yeast could be separated in a reasonable purity on a single column of silicic acid. A fractionation method, which consisted in utilizing silica gel and a gradient elution with the mixture of chloroform and methanol, has also been investigated in our laboratory. By this method separation into four fractions, phosphatidylethanolamine, a combined phosphatidylserine-phosphoinositide fraction, lecithin and sphingolipides, was made possible (*7*).

In the present paper an improvement in a column chromatographic

* This work was partly presented at the 31st Meeting of the Japanese Biochemical Society held in Sapporo, in July, 1958.

The following abbreviations are used: GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; GP, glycerophosphate; GPI, glycerophosphorylinositol; GPS, glycerophosphorylserine; GPG, glycerophosphorylglycerol; C-M, chloroform-methanol.

procedure is described, whereby the individual component of mixed phospholipides extracted from rat liver and heart can be separated on silica gel column.

EXPERIMENTAL

Reagents—Solvents, which were purified and distilled, were used for lipide extraction, fractionation, and paper chromatography.

Silica gel, was Kanto Chemical Company's reagent (for chromatographic use). Whenever possible, fresh bottles of silica gel were used. Otherwise, before use, the silica gel was dried at 105° for 12 hours before use. Toyo Roshi Company's Cellulose powder (mesh 100–200) was used for the removal of nitrogenous contamination.

Analytical Methods—Phosphorus was determined by Fiske-Subbarow's method (*8*), nitrogen by the micro-Kjeldahl procedure, glycerol by Blix's method (*9*), and choline by Entenman *et al.* method (*10*). Plasmal was determined by Leupold's method (*11*). The radioactivity was determined with a Geiger Müller tube, by plating aliquots on ceramic (or aluminium) planchets.

The following qualitative tests were carried out with the eluate: ninhydrin, Molisch, Scherer, plasmal and Liebermann-Burchard reactions.

The detections of phosphoric acid esters produced after mild alkaline hydrolysis of phospholipides in the individual eluate were made by the paper chromatographic procedure, in which the materials were developed with phenol saturated with 0.1 per cent ammonia and buthanol-propionic acid-water (2 : 1 : 1.3). The procedure have been described previously (*12*). Moreover, the detections of the constituents other than GP produced with acid hydrolysis of the phospholipides or phosphoric acid esters were also made by the paper chromatography. The development of these substances on filter paper were carried out with phenol saturated with 0.1 per cent ammonia and buthanol-acetic acid-water (4 : 1 : 2).

Extraction of Phospholipides—Twelve hours after intraperitoneal injection of P^{32} labeled orthophosphate (2.5 mc.) the rats were killed and the lipides in the liver and the heart were extracted separately with ethanol-ether (3 : 1). After grinding the tissues, about 20 volumes of the solvent were added. The extraction was carried out at 50° for thirty minutes. The mixture was filtered as rapidly as possible. Then the residues were extracted twice with 15 volumes of the same solvent system and then filtered. The filtrate combined was then evaporated to dryness under reduced pressure in a stream of nitrogen (below 50°). The residues were reextracted with 20 ml. of petroleum ether and 5 ml. of C–M (9 : 1).

The solvent was evaporated again to dryness and the residues were reextracted with chloroform. This solution was passed through a column packed with cellulose powder. By this treatment nitrogen containing contaminants were removed. Chloroform was evaporated and the residues were extracted with ether. This ether solution was used as the material for the following column chromatographic fractionation.

Preparation of Column—2.2 g. of silica gel was packed into a glass tube (1 cm. in diameter) to give a bed height of 7 cm. The column was washed successively with 7 ml. of methanol, ether and petroleum ether (b. p. 60–70°). The flow rate was kept at 1 ml. per minute by application of a slight pressure of nitrogen, if necessary.

RESULT

Chromatographic Procedure—After the silica gel had reached a constant level and petroleum ether was allowed to drain nearly to the surface of the silica gel, the sample was poured into the column so as not to disturb the surface. About 70 mg. of the samples were used for the liver lipides and about 20 mg. for the heart lipides. When the solvent flowed down to the surface, 20 ml. of ether-methanol (8 : 1) were added slowly into the column, which was then attached to the eluate receiver, and elution was carried out. By this procedure the acetone soluble lipides (ester-cholesterol, fat, free cholesterol) are removed from the column.

Then, the subsequent elution of the phospholipides still remaining in the column was conducted with chloroform and methanol. The solvent was generally used in the following order: C-M (9 : 1) (*v/v*), C-M (4 : 1), C-M (3 : 1), C-M (3 : 2), C-M (1 : 1) and methanol. The size of the fractions collected was 4 ml. In the experiments, the progress of the fractionation was followed by radioactivity determination* on each individual tube and, when it was necessary to change solvent system, the new solvent was added after the previous solvent had reached the surface of the silica gel.

Liver Phospholipides—The elution curve are shown in Fig. 1. The fraction eluted with C-M (9 : 1) was designated as Fr. I.

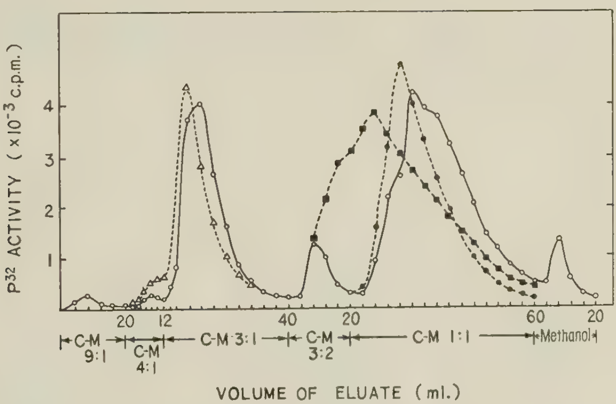

FIG. 1. Chromatogram of rat liver-mixed phospholipides, on silica gel column.

---△--- indicates elution curve with C–M (3 : 1) soon after elution with C-M (9 : 1), ---■--- indicates elution curve with C–M (1 : 1) soon after elution with C–M (3 : 1), ---●--- indicates elution curve with C–M (1 : 2) soon after elution with C–M (3 : 2).

* Twelve hours after intraperitoneal injection of P^{32} labeled orthophosphate the specific radioactivity of the individual phospholipide had been shown to be equal. (*12*). Therefore, the radioactivity is pararell to the lipide-P and then the activity curve accords with the lipide-P curve.

In the elution with C-M (4 : 1) the size of the fractions collected was 2 ml. This C-M (4 : 1) fraction was designated as Fr. II. and the following C-M (3 : 1) fraction as Fr. III.

If the eluting solvent was replaced with C-M (3 : 1), soon after the elution with C-M (9 : 1), the peak found with C-M (4 : 1) elution was not clearly recognized as shown with the dotted line. Therefore, it was thought that Fr. II was inseparable from Fr. III, when the eluting solvent was changed directly from C-M (9 : 1) to C-M (3 : 1).

As the activity did not change after the tenth tube, so the solvent system was replaced with C-M (3 : 2) on the twelfth tube. The elution curve with C-M (3 : 2) showed also a peak. The highest activity was already found in the second tube. Then, it decreased gradually. However, the activity increased again from the seventh tube. This fraction eluted with C-M (3 : 2) to the sixth tube was designated as Fr. IV. When the elution continued with C-M (3 : 2), the activity increased to the tenth tube and then decreased very slowly. The significant activity was found still more after the elution with 100 ml. of this solvent system and a large quantity of solvent was necessary to obtain the eluate showing slight activity. However, if the solvent system was replaced with C-M (1 : 1), the lipide was eluted more rapidly than C-M (3 : 2). The activity in the eluate increased sharply to the tenth tube and then showed a fairly sharp decline. This C-M (1 : 1) fraction designated as Fr. V.

The solvent was replaced with methanol on the seventeenth tube. In this elution a peak was found. The activity was highest in the second tube. This fraction was expressed Fr. VI.

When C-M (1 : 1) was used as the eluting solvent system soon after C-M

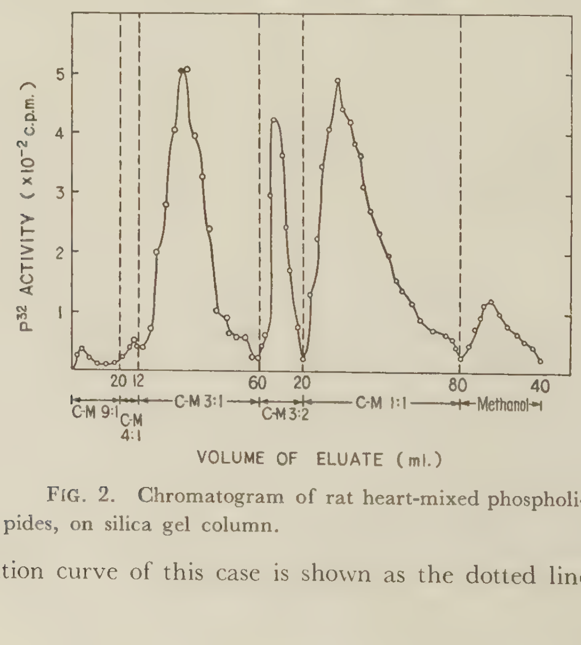

FIG. 2. Chromatogram of rat heart-mixed phospholipides, on silica gel column.

(3 : 1), the elution curve of this case is shown as the dotted line. In this case

Fr. IV was inseparable from Fr. V. If the elution with C-M (1:2) was employed after C-M (3:2), the elution curve as shown in Fig. 2 was obtained. In this case it was noticed that Fr. VI was inseparable from Fr. V, because the activity contained in the original sample had been already recovered, though the peak of Fr. VI was not found.

These results show that the phospholipides from the rat liver can be separated in six fractions by this column chromatographic procedure on silica gel.

The reproducibility of this fractionation was excellent, as is shown in the data of the distribution of phospholipide in the eluates, which follows:

Fr.	I	1–1.5 per cent	(1 per cent)
	II	1–3 ,,	(2 ,,)
	III	27–28 ,,	(28 ,,)
	IV	5–6 ,,	(6 ,,)
	V	56–59 ,,	(57 ,,)
	VI	5–7 ,,	(5 ,,)
Total			99 per cent

Heart Phospholipides—Fractionation of the phospholipides extracted from the heart was carried out with the same solvent system as in the separation of the liver phospholipides. The elution curve was shown in Fig. 2. The phospholipides from the heart were separated into six fractions. The distribution of the phospholipides in the eluates was as follows:

Fr.	I	1 per cent
	II	1 ,,
	III	36 ,,
	IV	13 ,,
	V	42 ,,
	VI	7 ,,
Total		100 per cent

Chemical Nature of Eluate Fractions—As shown in Figs. 1. and 2. the phospholipides were separated into six fractions. The results of quantitative analysis and qualitative tests of the individual fraction are shown in Table I and II and Fig. 3.

The initial fraction (Fr. I) was composed of a high glycerol-containing component (Glycerol : P=1.25). The autoradiogram after mild alkaline hydrolysis of the eluent shows that the main lipide in Fr. I is an unknown phospholipide. R_f value of the hydrolysate accords with GPG obtained as the hydrolysate of phosphatidylglycerol the presence of which in plant tissue have been described by Benson and Maruo (*13*). However, the existence of this lipide in the animal tissues has not yet been reported up to the present. Meanwhile, the phospholipides containing polyglycerophosphate have been reported by Pangborn (cardiolipin) (*14*), Fleury (glycèro-phosphatogène) (*15*), McKibbin and Taylor (polyglycerophosphatide) (*3*)

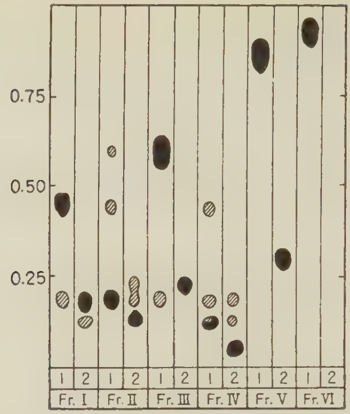

FIG. 3. Autoradiogram of mild alkaline hydrolysates of liver phospholipides eluated.
Solvent system: 1. Phenol-NH_3 (0.1 per cent), ascending, 14 hours.
2. Buthanol-propionic acid-H_2O, ascending, 12 hours.

TABLE I

Qualitative Analysis and Quantitative Tests of Liver Phospholipides Eluted into Individual Fraction by Various Solvent System

	C-M (9:1) I	C-M (4:1) II	C-M (3:1) III	C-M (3:2) IV	C-M (1:1) V	Met OH VI
P (%)	3.81	3.70	3.65	3.51	3.8	3.95
N (%)	—	—	1.38	—	1.80	2.48
Glycerol (%)	14.2	9.7	10.1	9.1	10.2	2.93
Choline (%)	—	—	0.0	0.0	16.5	14.0
N : P	—	—	0.84	—	1.05	1.4
Glycerol : P	1.25	0.88	0.93	0.88	0.91	0.25
Choline : P	—	—	0.0	0.0	0.97	0.77
Ninhydrin test	+	++	+++	++	—	+
Molisch "	±	—	—	+	—	—
Scherer "	—	—	—	++	—	—
Acetal	++++	+	++	+	++	—
Liebermann-Burchard	—	—	—	—	—	—

TABLE II

Qualitative Tests of Heart Phospholipides Eluted into Individual Fraction by Various Solvent System

Color test \ Fraction	C-M (9 : 1) I	C-M (4 : 1) II	C-M (3 : 1) III	C-M (3 : 2) IV	C-M (1 : 1) W	Met OH VI
Ninhydrin	+	++	+++	++	+	++
Molisch	—	—	—	+	—	—
Scherer	—	—	—	+	—	—
Acetal	+	++	++++	++	+++	++
Liebermann-Burchard	—	—	—	—	—	—

and Igarashi *et al.* (*16*). Therefore, further works* are required for identification of this lipide. In the autoradiogram another spot which showed low activity was found. It accorded with GPS. Then, it was recognized that Fr. I contained two phospholipides, namely, a phospholipide having high glycerol content as the main component and small amounts of phosphatidylserine. Furthermore, of interest was the finding that plasmal reaction was the most intensive in Fr. I of the liver, though it was also found to be positive in Fr. II, III, IV and V. In the heart, however, plasmal reaction was the most intense in Fr. III and was weak in Fr. I. These results shows that some types of acetal phospholipide other than ethanolamine or choline derivatives, exist in animal tissues and the content of fatty aldehyde is the higest in Fr. I (a high glycerol containing phospholipide fraction) in the liver, while in the heart it is higher in Fr. III (phosphatidylethanolamine fraction) than in other fractions.

The second fraction (Fr. II) was shown to contain phosphatidylserine as the main component by the autoradiogram. Three radioactive spots were detected on the paper developed after mild alkaline hydrolysis. Their Rf values accorded with those of GPS, GPE and GPG. However, nearly all the activity localized in GPS., showing that this fraction mainly consisted of phosphatidylserine.

The phospholipide in the eluate Fr. III was found to be phosphatidylethanolamine by the autoradiogram after mild alkaline hydrolysis. The autoradiogram showed that nearly all its radioactivity based on GPE. Only

* In the proceeding experiments with large amounts of ox liver phospholipides the following data is obtained for Fr. I: P, 3.87 per cent; N, 0, 0.56 per cent; glycerol, 13.9 per cent; acetal, 9.95 per cent.

The nitrogen content decreases to a negligible degree by rechromatography of the eluted lipides in Fr. I with C-M (95 : 5) on silica gel, but the increase in glycerol content is small. Therefore, the main component of Fr. I is a polyglycerophospholipide.

slight activity was found on area accorded to GPS. Then, the phospholipide in this fraction is phosphatidylethanolamine.

Fr. IV was the only fraction which showed positive Scherer test. The autoradiogram of mild alkaline hydrolysate of the eluted phospholipide showed three spots containing radioactivity. The highest activity was found in the spot accorded with GPI. The other two spots showing very low activity accorded with GPS and GPG respectively. From these results it was suggested that the main phospholipide eluted into this fraction is inositol containing phospholipide.

Fr. V was the first choline-positive fraction. From the analytical data it was supposed that this fraction composed of phosphatidylcholine. The autoradiogram showed also a single spot which contained radioactivity. Its R_f accorded with GPC. These results indicate that the phospholipide eluted with C-M (1 : 1) was phosphatidylcholine.

Fr. VI contained also phospholipides. The analytical data in Table I show that this fraction has the highest nitrogen and lowest glycerol content. The ratios of choline to phosphorus, glycerol to phosphorus and nitrogen to phosphorus were 0.93, 0.25 and 1.40 respectively. From these results it was suggested that this fraction contained two phospholipides, one of them being sphingomyelin-like phospholipide of high nitrogen content and the other phosphatidylcholine. The autoradiogram showed the existence of GPC, but sphingosylphosphoryl compounds could not be detected as a spot. This may be due to the fact that most sphingolipides are not hydrolysed by the mild alkaline hydrolysis above described and so these lipides are transferred to the organic solvents, thus escaping detection.

DISCUSSION

In the previous report the fact that the phospholipides mixture can be separated into four fractions by a column chromatographic method has been presented. In the present investigation the method has been studied in detail and a satisfactory separation of many of the components of the phospholipides from rat liver and heart can be achieved through column chromatographic procedure on silica gel. In effect with varying mixtures of chloroform-methanol (9 : 1 ; 4 : 1 ; 3 : 1 ; 3 : 2 ; 1 : 1 and 0 : 10), the phospholipides can be separated into six fractions, namely a phospholipide of high glycerol and low nitrogen content, the phosphatidylserine, the phosphatidylethanolamine, the inositol containing phospholipides, the phosphatidylcholine and a combined phosphatidylcholine-sphinogolipides fraction. The elution pattern obtained was reproducible. The percentage of the phospholipide eluted into the individual fraction was constant for the liver and the heart respectively, though the values varied to a small extent according to the kind of the tissue examined. The lipide phosphorus applied to a column can be recovered in good yields for the phospholipides from the liver and heart. This procedure, however, may not be applicable to every source of phospholipides.

The initial fraction contained a phospholipide of high glycerol and low nitrogen content. The lipide is not clearly identified at present. Analytical data suggest that it is not phosphatidylglycerol, but a polyglycerophospholipide, though R_f value of the mild alkaline hydrolysate on the autoradiogram is in accord with that of GPG. It has already been known that the content of the acetal phospholipides was small in the liver. In the present study it was demonstrated that its largest part was concentrated in the initial fraction, though plasmal reaction was positive in Fr. II, III, IV and V respectively. On the other hand, the acetal phospholipides in the heart distributed as the ethanolamine and choline derivatives for the most part.

The major components, phosphatidylcholine and phosphatidylethanolamine, can be separated in highly purified form on a single column chromatography, although these were contaminated with very small amounts of other phospholipides.

The minor components were also prepared in comparatively purified form. The most part of the inositol-containing phospholipides were eluted in Fr. IV. Previously the fact that the inositol containing phospholipides are eluted subsequent to phosphatidylethanolamine and preceding phosphatidylcholine was presented by us. Hanahan *et al.* (*6*) indicated the same result. These finding offer a useful tool for the isolation of inositol containing phospholipides instead of the classical solvent method of Folch (*17*).

Thus the fractionation of the mixed phospholipides into the individual component in purified form could be achieved with good recovery. Applying this method, the isolation of the individual components, especially the phospholipides in Fr. I and IV, from large quantities of the liver and the heart are now proceding. The details of this investigation will be reported in forthcoming publication.

SUMMARY

The column chromatographic separation of the mixed phospholipides from rat liver and heart on silica gel was described. Through the use of various mixtures (*v/v*) of chloroform-methanol (9 : 1 ; 4 : 1 ; 3 : 1 ; 3 : 2 ; 1 : 1) and methanol the phospholipides can be separated into six fractions, namely a high glycerol, low nitrogen containing phospholipide, phosphatidylserine, phosphatidylethanolamine, phosphoinositides, phosphatidylcholine, a combined phosphatidylcholine-sphingolipides fraction. It is also known that plasmal reaction is the most intense in Fr. I of the liver, though it is found to be positive in other fractions. Thus, it may be thought that the high glycerol containing acetal phospholipids is larger in amount than other acetal phospholipides in the liver. In the heart tissue, however, the acetal phospholipides distributed as the ethanolamine and choline derivatives for the most part.

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A SIMPLE METHOD FOR PURIFICATION OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE*

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In the previous paper (*1*) it has been shown that small amounts of the mixed phospholipides isolated from rat liver were separated into six fractions and both phosphatidylserine and phosphatidylethanolamine could be obtained in highly purified form by a single column chromatography on silica gel. Then, the column chromatographic separation was followed by large quantities of mixed beef liver phospholipides.

In the present paper it is described that phosphatidylserine and phosphatidylethanolamine are also eluted with chloroform-methanol (4:1) and (3:1) separately and are able to be purified easily by rechromatography on silica gel.

EXPERIMENTAL

Reagents—All solvents were purified and redistilled before use for lipide extraction and fractionation. Silica gel was Kanto Chemical Company's reagent (for chromatographic use), and a fresh one should be always used as bottles is opened, otherwise, the silica gel was dried for 12 hours at 105° before use. Cellulose powder mesh 100-200, was a product of Toyo Roshi Kaisha, Ltd.

Methods—Phosphorus was determined dy Fiske-Subbarow's method (*2*), nitrogen by the micro-Kjeldahl procedure, glycerol by Blix' method (*3*), choline by Entenman *et al.* method (*4*), plasmal by Leupold's method (*5*) and iodine number by Yasuda's method (*6*). Total fatty acids were isolated by ether extraction from hydrolysate with hydrochloric acid, washed well with water, desiccated with anhydrous sodium sulfate and processed by the usual manner. GP** was separated as barium salt and identified by both the chemical analysis and paper chromatography. GPS and GPE, the mild alkaline hydrolysis products of phosphatidylserine and phosphatidylethanolamine, were separately obtained as barium salt and identified by paper chromatography. The bases, serine and ethanolamine, were identified by paper chromatographic procedure.

* This work was partly presented at the 31st Meeting of the Japanese Biochemical Society held in Sapporo, in July, 1958.

** The following abbreviations are used in this report: GP, glycerophosphate, GPS, glycerophosphorylserine, GPE, glycerophosphorylethanolamine, GPG, glycerophosphorylglycerol, DNFB, dinitrofluorobenzene, DNP, dinitrophenol, C-M, chloroform-methanol.

They were also isolated as DNP-derivatives and compared with the authentic DNP-serine and DNP-ethanolamine respectively, which were prepared from DL-serine and ethanolamine hydrochloride. These bases and other ninhydrin reactive substances were detected qualitatively by paper chromatographic procedure. Carbohydrates were detected by Molisch reaction, sterols by the Libermann-Burchard test and inositol by Scherer's test.

ISOLATION OF PHOSPHOLIPIDES

About 2 kg. of beef liver were minced and dehydrated with acetone for a day and then filtered. The residues were extracted with 95 per cent ethanol-ether (3 : 1) mixture at 50° for thirty minutes and then filtered. The residues were reextracted with the same solvent system and then filtered. For the lipide extraction 50 volumes of the solvent were used. The filtrate combined was then evaporated to dryness under reduced pressure in a nitrogen stream. The residues were reextracted with ether. Then, the ether extracts were concentrated to a small volume, to which a 10 volumes of acetone were added. The mixture was kept in a refrigerator overnight. Acetone soluble lipides were removed and the acetoneinsoluble lipides were washed several times with acetone and dried *in vacuo*. The material thus obtained was 66 g. The ether solution of this substance was applied to the following column chromatography.

Isolation of Crude Phosphatidylserine and Phosphatidylethanolamine

Preparation of Columns—One hundred and ten g. of the silica gel per 5 g. of phospholipides to be applied were packed in the column (5 cm in diameter) to give a bed height of 12 cm. Then, the column was washed with 200 ml. of methanol, and then 200 ml. of ether. The flow rate of 6 to 8 ml. per minute, was maintained, if necessary, by a slight nitrogen pressure.

Chromatographic Procedure—When the level of washing solvent proceeded down nearly to the surface of silica gel layer, 5 g. of the sample dissolved in 250 ml. of ether was placed on to the column so as not to disturb the surface. When the solvent was decreased to drain nearly to the surface, ether-methanol (8 : 1) were added slowly into the column and the column was attached to the eluate receiver. Three hundred ml. of this solvent mixture were poured into the column. Then, the separation of phospholipides was carried out with mixture (*v/v*) of chloroform-methanol. The elution was carried out with the following order: C-M (9 : 1) 500 ml. (Fr. I), C-M (4 : 1) 300 ml. (Fr. II), C-M (3 : 1) 1000 ml. (Fr. III), C-M (3 : 2) 500 ml. (Fr. IV), C-M (1 : 1) 1500 ml. (Fr. V) and methanol 500 ml.

The content of lipide-phosphorus eluted into the individual fraction was shown in Table I.

As it is considered that phosphatidylserine is eluted mainly into Fr. II and phosphatidylethanolamine into Fr. III based on the previous experiments (*1*), the analysis for these fractions were performed. The data is shown in Table II. The results showed that the phospholipides eluted into Fr. II and III containing none of choline. Scherer test was negative. The mild alkaline

TABLE I

The Content of Lipide-Phosphorus Eluted into the Individual Fraction

	Eluting solvent		Lipid-P	
			(mg.)	(%)
0	Ether : methanol	(8 : 1)	0.0	0.0
I	Chloroform : methanol	(9 : 1)	70.5	2.7
II	,, : ,,	(4 : 1)	108.5	4.1
III	,, : ,,	(3 : 1)	544.0	21.0
IV	,, : ,,	(3 : 2)	288.0	11.0
V	,, : ,,	(1 : 1)	1400.0	53.0
VI	Methanol		240.0	8.0

TABLE II

Quantitative Analysis of Fractions Obtained from Chromatogram of Beef liver Phospholipids on Silica Gel

	Fr. II	Fr. III
P (%)	3.76	3.72
N (%)	1.63	1.66
N : P (molar ratio)	0.97	0.99
Glycerol (%)	10.9	10.5
Gly. : P (molar ratio)	0.98	0.95
Choline (%)	0	0
Sugar (%)	—	—
Fatty acid (%)	53.0	61.6
Fatty acid[1)] : P (molar ratio)	1.53	1.81
Aldehyde (%)	0.20	0.22
Iodine Number	57	84

1) In order to calculate the molar ratio of its, fatty acid was considered as oleic acid (molecular weight 282).

hydrolysates of Fr. II and III lipides were detected separately by paper chromatographic procedure. A few spots of phosphorus compounds were

found on the chromatogram, though GPS and GPE were recognized as the main substances in the hydrolysates of Fr. II and Fr. III respectively.

Rechromatography of Crude Phosphatidylserine—In order to purify, a re-chromatography on silica gel was carried out.

The crude preparation (2.7 g.) were dissolved in 75 ml. of C-M (9 : 1). Ten g. of the silica gel were packed into the column (2.2 cm. in diameter). Then, the column was washed with 50 ml. of C-M (9 : 1). When the solvent was decreased to drain nearly to the surface, the sample was poured carefully. The elution was performed with the following solvent system successively: C-M (9 : 1) 50 ml., C-M (4 : 1) 100 ml., and C-M (3 : 1) 60 ml. The elution curve are shown in Fig. 1.

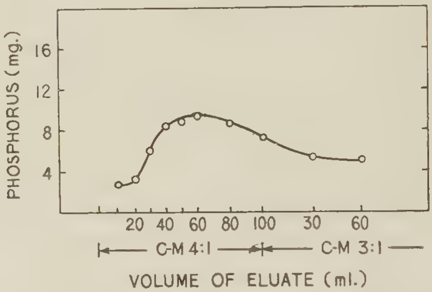

FIG. 1. Rechromatography of fraction (4 : 1) by silica gel column.

The eluate with C-M (4 : 1) (20–100 ml.) was combined and the solvent was evaporated to dryness under reduced pressure in a nitrogen stream. The residues were redissolved in 30 ml. of chloroform. This solution was passed through a column (2.2 cm. in diameter) packed with 20 g. of cellulose powder and washed with 100 ml. of chloroform. The eluted solution was evaporated and the residues were dried *in vacuo*. The yield of the substance thus obtained was 1.6 g. This substance was readily soluble in chloroforn, soluble in ether and insoluble in acetone.

Hydrolysis of Phosphatidylserine—The material thus obtained was hydrolyzed with acid and alkali separately.

(*1*) *Acid Hydrolysis*: The preparation (0.5 g.) free from solvent were hydrolyzed in a boiling water bath for 4 hours with 10 ml. of 3 *N* HCl. The hydrolysate was extracted with 50 ml. of ether in four portions. The aqueous phase was decolorized with charcoal and concentrated to dryness under reduced pressure on a warm water bath. The residues (140 mg.) were redissolved in 5 ml. of water. Then, solid $Ba(OH)_2$ was added till the solution is alkaline. Precipitate appeared which was centrifuged and the clear supernatant was decanted. To the supernatant were added an equal volume of absolute ethanol. Immediately barium glycerophosphate precipitated. After standing for several hours the mixture was centrifuged and the super-

natant was decanted. The yield of barium glycerophosphate was 93 mg. The excess of $Ba(OH)_2$ was removed from the supernatant as barium carbonate. Then, the solution was concentrated dryness. The weight was 57 mg. This preparation was detected by paper chromatography with phenol saturated with 0.1 per cent ammonia and buthanol-acetic acid-water (4 : 1 : 2). A single spot was found on each chromatogram and its R_f accorded with that of serine. The DNP derivative, prepared by the method of Sanger (*7*), recrystallized from methanol has m.p. 174°, which is in accord with that of the DNP-L-serine. Furthermore, the absorption curve of this compound was identical with that of authentic DNP-serine.

The ether layer of the acid hydrolysate was evaporated to dryness under reduced pressure in a nitrogen stream. The residues were dried *in vacuo*. The yield was 250 mg. These fatty acids consisted of 28.3 per cent of solid acids (m.p. 62-65°) and 71.7 per cent of liquid acids.

(*2*) *Mild Alkaline Hydrolysis*: One g. of the preparation was hydrolyzed with 0.2 N NaOH in a mixture* of chloroform, methanol and water at 37° for thirty minutes. Then, 160 ml. of ice-cold water were added. The milky solution was passed through a column packed with Amberlite IRC 50. The column was washed with water thoroughly. The slightly acidic eluate was extracted with 200 ml. each of chloroform, ether, petroleum ether and 300 ml. of isobutanol successively. The aqueous layer was neutralized with dilute ammonium hydroxide and evaporated to dryness under reduced pressure below 50°. The residues were dissolved in 5 ml. of water. A portion of this solution was subjected to paper chromatography. Only a single spot was detected on the chromatograms developed with phenol saturated with 0.1 per cent ammonia water and butanol-propionic acid-water (2 : 1 : 1.3) by ninhydrin and molybden reagents, the R_f value being identical with that of GPS. Then, the following procedures were carried out on the greater part of the solution. Solid barium hydroxide was added to the solution. None of precipitate did appeared. Then, an equal volume of ethanol was added to it. None precipitate was produced. When additional 5 ml. of ethanol was added, white precipitate was produced. After standing overnight in a refrigerator, the precipitate was centrifuged and washed twice with ethanol-water (2 : 1). After drying in vacuum, the substance weighed 150 mg.

After barium was removed as barium carbonate, the material was hydrolyzed in a boiling water bath for thirty minutes with 1 N HCl. Then, the hydrolysate was evaporated to dryness under reduced pressure and hydrochloric acid was thoroughly removed. The residues were dissolved in a small amount of water. Solid barium hydroxide was added to the solution. Then, an equal volume of ethanol was added. Precipitates of barium glycerophosphate appeared which was centrifuged and dried in vacuo. From the filtrate the excess of barium hydroxide was removed by centrifugation as barium carbonate. The filtrate was concentrated and used for paper

* $CHCl_3$ 50 ml., MeOH 25 ml., H_2O 5 ml., and 1 *n* $NaOH/CH_3OH$ 20 ml.

chromatographic procedure. Only one spot, which was positive with ninhydrin reagent, appeared on paper chromatogram, developed separately with phenol saturated with 0.1 per cent ammonia water and *n*-butanol-acetic acid-water (4 : 1 : 2). Its R_f accorded with that of serine. The yield of this substance was 35 mg. Phosphorus containing substances were not detected on the chromatograms. Sugars were not also detected.

Rechromatography of Crude Phosphatidylethanolamine—The crude preparation (10 g.) were dissolved in 200 ml. of chloroform. Silica gel (100 g.) was packed into the column (50 cm. in diameter) and washed with C-M (9 : 1) 100 ml. and then C-M (4 : 1) 100 ml. After the sample was poured into the column, the elution was performed with the following solvent system successively: C-M (9 : 1) 100 ml., C-M (4 : 1) 300 ml., and C-M (3 : 1) 500 ml. The elution curve are shown in Fig. 2.

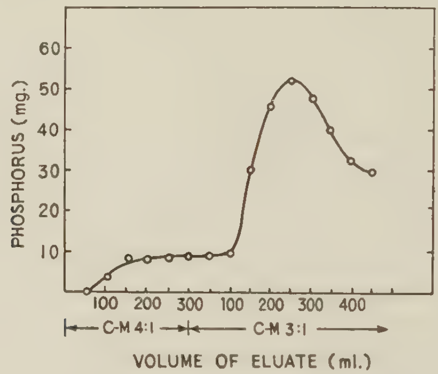

FIG. 2. Rechromatography of fraction (3 : 1) by silica gel column.

The eluate with C-M (3 : 1) (150–450 ml.) was combined and the solvent was evaporated to dryness under reduced pressure in a nitrogen stream. The residues were redissolved in 200 ml. of chloroform. This solution was passed through a column (2.2 cm. in diameter) packed with 20 g. of cellulose powder and washed with 300 ml. of chloroform. The effluent was evaporated and the residues were dried *in vacuo*, which weighed 6.0 g. This substance was soluble in ether, chloroform and ethanol, insoluble in acetone.

Hydrolysis of Phosphatidylethanolamine—The preparation eluted with C-M (3 : 1) by rechromatography on silica gel was hydrolysed with acid and alkali separately.

(*1*) *Acid Hydrolysis*: One g. of the sample were hydrolyzed in a boiled water bath for 4 hours with 10 ml. of 3 *N* HCl. The hydrolysate was extracted with 100 ml. of ether in four portions. The aqueous layer was decolored with charcoal and concentrated to dryness under reduced pressure. The residues were dissolved in 5 ml. of water and then solid barium hydroxide was added. The precipitate appeared was removed by centrifugation. To the clear supernatant were added an equal volume of absolute ethanol.

Immediately white precipitate appeared. After standing several hours it was removed by centrifugation and dried in vacuum. The weight was 150 mg. It was shown to be barium glycerophosphate by analytical and paper chromatographic results.

Excess of barium hydroxide was removed from the supernatant as barium carbonate. The filtrate was evaporated to dryness under reduced pressure. The yield was 70 mg. A small amount of this material was applied to paper chromatography. Only one ninhydrin positive spot was found on the paper chromatograms developed with phenol saturated with 0.1 per cent ammonia water and *n*-butanol-acetic acid-water (4 : 1 : 2). R_f of the spot accorded with that of ethanolamine. Its DNP-derivative, prepared by the method of Axelrod (*8*) and recrystallized twice from ether and dried *in vacuo*, melted sharply at 90°, and thus was identified as DNP-ethanolamine. Furthermore, the absorption curve of this compound was identical with that of authentic DNP-ethanolamine.

The ether layer of the acid hydrolysate was evaporated to dryness under reduced pressure in a nitrogen stream. The residue was dried *in vacuo*. The yield was 616 mg. The fatty acids were consisted of 19.7 per cent of solid acids (m.p. 68–69°) and 80.3 per cent of liquid acids.

(*2*) *Mild Alkaline Hydrolysis*: One g. of the preparation was used. The procedure was the same as described in phosphatidylserine. After hydrolysis, removal of alkali by Amberlite IRC 50 and extraction with organic solvents, the water phase was neutralized with dilute ammonium hydroxide and evaporated to dryness under reduced pressure below 50°. By paper chromatography of this solution it was shown that the substance contained in this solution was only one and it was GPE. Then, solid barium hydroxide was added and ethanol was added until precipitation occurred. After standing overnight in a refrigerator, the white precipitate was centrifuged and washed twice with ethanol-water (2 : 1). After drying *in vacuo*, it weighed 150 mg.

After barium removed as barium carbonate, it was hydrolyzed with 1 *N* HCl. The hydrolysate was evaporated to dryness under reduced pressure and hydrochloric acid was thoroughly removed. The residues were dissolved in a little water. Solid barium hydroxide was added to the solution. Then, an equal volume of ethanol was added. The white precipitate was centrifuged and dried *in vacuo*. From the supernatant the excess of barium hydroxide was removed by centrifugation as barium carbonate. The filtrate was evaporated to dryness under reduced pressure below 50°. The residues were dried *in vacuo*. This weighed 35 mg. Its paper chromatogram showed that this substance was ethanolamine. Phosphorus containing substances and sugars were not detected.

DISCUSSION

The isolation of phosphatidylethanolamine from the animal tissues had been carried out by many investigators up to the present. Majority of the

methods, however, were complicated and the preparations were not always pure. Recently it was found that column chromatographic procedure is a useful tool for the isolation of various lipides. Hanahan *et al.* (*9*) and Sakagami *et al.* (*1*) reported that the mixed-phospholipides from the animal tissues were separated into several fractions and phosphatidylethanolamine and phosphatidylserine could be obtained in highly purified form by a single chromatographic procedure on silicic acid or silica gel column using comparatively small amounts of lipides. In the present experiments the column chromatographic separation of large amounts of mixed-phospholipides from beef liver was tried. The results showed that large amounts of phospholipides could also be separated into the same fractions as found in the case of small amounts. Then, in order to purify phosphatidylserine and phosphatidylethanolamine thoroughly, rechromatography of these substances were carried out. From the results as shown above it was found that phosphatidylserine and phosphatidylethanolamine were absolutely purified by the procedure and obtained in good yield. Thus, it is thought that this method is a simple and convenient one to purify these phospholipides and to obtain in good yield.

SUMMARY

By use of large amounts of mixed-phospholipides from beef liver, a column chromatographic separation was investigated. The results showed that large amounts of mixed-phospholipides could also be separated into the same fractions as obtained in the small scale chromatography.

Though phosphatidylserine was mainly eluted with C-M (4 : 1) and phosphatidylethanolamine mainly with (3 : 1), they were contaminated with other lipides. Therefore, in order to purify these lipides rechromatography was carried out. Acid hydrolysis and mild alkaline hydrolysis showed that phosphatidylserine and phosphatidylethanolamine could be highly absolutely purified by the procedure in good yield.

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STUDIES ON THE RIBONUCLEOPROTEIN PARTICLES

V. EFFECT OF ALKALI AND SALINE ON THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES

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The bonding between nucleic acid and protein in the deoxyribonucleoprotein (DNP) molecule seems to have been established as an electrostatic one (*1*), while there have been some indications that that in ribonucleoprotein (RNP) molecule is not purely electrostatic, hydrogen bonds being probably involved. Proving that ribonucleic acid (RNA) can be isolated from mammalian tissues by treatment with phenol, Kirby (*2*) concluded that RNA-protein link would be of hydrogen bond nature, which were disrupted by phenol, a hydrogen bond forming compound. In 1958, Elson (*3*) reported the dissociation of RNP by urea treatment and arrived at a similar conclusion.

Recently the present authors* have examined the effect of various hydrogen bond forming compounds on the RNP isolated from the rat liver microsomes and found that, beside phenol, *o*-cresol can isolate RNA from the RNP. In spite of the apparent homogeneity of the electrophoretic patterns, however, these RNAs were contaminated with considerable amount of protein. On the other hand, other hydrogen bond forming compounds such as pyridine, picoline, *etc.* were found hardly effective in dissociating RNA from the RNP molecules. These results appear to suggest a possibility that the RNP contains RNA and protein linked together by bonds other than hydrogen bonds. Therefore, the effect of alkali and strong saline on the dissociation of microsomal RNP were studied and the question as to whether electrostatic bonding is really concerned or not was examined.**

EXPERIMENTALS

Isolation of RNP—The RNP was isolated from the rat liver microsomes with sodium deoxycholate as previously described (*4*). All the isolation procedures were performed in the cold (0–5°).

Electrophoretic Analysis—Effect of alkaline pH on the RNP was observed at 4° by Tiselius' electrophoretic method (Hitachi Model HTD electrophoretic Apparatus). The RNP solutions were previously dialysed overnight in the cold against the various Miller-Golder's buffer solutions of ionic strength 0.1 (*5*), their pH being 7.0, 9.5, 10.0, 11.0 or,

* J. Biochem., in press.

** A preliminary account of this work has been published. (*Kagaku*, **29**, 365 (1659))

12.0 respectively. After electrophoresis, the sample was pipetted out of the cell, and its content of RNA and protein was determined. Conductivity of the buffer solution was measured at 0° by Wheatstone bridge method and the electrophoretic mobility at 0° was calculated.

Differential Centrifugation—Effects of saline on the RNP was examined by the differential centrifugation (Hitachi Model 40 P Ultracentrifuge), for it is very difficult to perform electrophoretic analysis in strong saline of high conductivity.

To each centrifuge tube containing 1 ml. of the RNP solution (its concentration was about 0.5 per cent), 5 ml. of neutral saline was added, concentration of which was properly adjusted to obtain final NaCl concentration of 0.1 (control), 0.5, 1.0, 1.5, 2.0 and 3.0 M respectively. PH of added saline had been previously adjusted to 7.0 by phosphate buffer. It is desirable to use a concentrated RNP solution, because of concentration dependency of salting out of RNA in saline. In our experiment, about 0.5 per cent RNP solution was used, hence RNP concentration after the addition of saline was about 0.08 per cent.

Four hours or 24 hours after standing still in the cold, they were subjected to the differential centrifugation following the scheme outlined below, all the supernatant being carefully separated by decantation and sediments were analysed on RNA and protein.

RNP+NaCl

2,500×g, 20 minutes

Sd-1 Sup-1

20,000×g, 20 minutes

Sd-2 Sup-2

105,000×g, 120 minutes

Sd-3 Sup-3

+perchloric acid (final concentration 0·5 M)

2,500×g, 20 minutes

Sd-4 Sup-4

In order to perform high speed centrifugation at 105,000×g safely, it is required for the centrifuge tube to be filled with any liquid, so that about 4 ml. of liquid paraffine was layered on the Sup-2 solution to fill the dead space. Great cares were paid for decantation of the Sup-3, not to let the sediment (Sd-3) flow because of its loosely packed state. All these procedures were carried out in the cold.

Analysis of RNA and Protein—The Schmidt-Thannhauser's method slightly modified by Littlefield *et al.* (*6*) was employed throughout. RNA content of the samples was determined from the optical density at 260 mμ of acidified NaOH extract (Hitachi EPV-2 Spectrophotometer) by employing a value of 34.2 per mg. per ml. per cm. as its extinction coefficient. The precipitate from the acidified NaOH extract was dissolved in N NaOH by incubating it at 37° overnight. Then, the amount of protein was determined by Lowry's method (*7*). The colour developed by Folin's reagent was not strictly proportional to protein concentration. Hence an empirical calibration curve was used throughout, which was constructed by taking the dry weight of protein (dried over 5 hours at 110°) in an aliquot of the RNP solution as a standard.

RESULTS

A. Effects of Alkali on the RNP Particles

1. Electrophoretic Patterns of the RNP in Neutral and Alkaline Media—A typical example of the electrophoretic patterns of the RNP in neutral and alkaline media is illustrated in Fig. 1 (a-f), in which the patterns of 1 (a) would

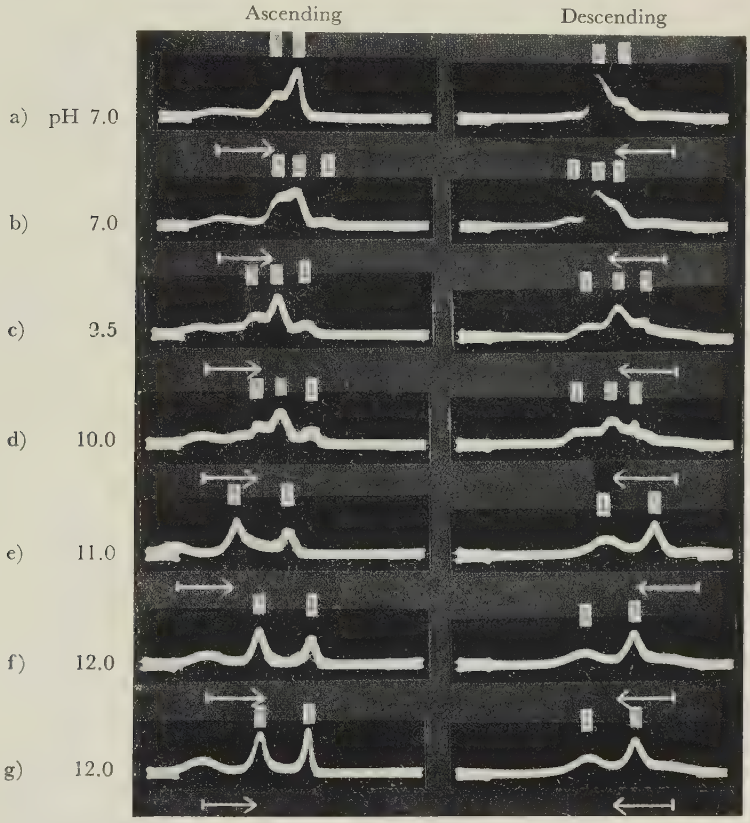

FIG 1. Effect of alkali on the electrophoretic patterns of the microsomal RNP.

The RNP solutions were dialysed overnight against (a) the "buffered dialysate" of the microsomal supernant, or against the Miller-Golder's buffer of ionic strength 0.1 at (b) pH 7.0, (c) 9.5, (d) 10.0, (e) 11.0, or (f) 12.0 respectively in the cold, and analysed by a Tiselius' electrophoretic method at 4°. The RNP solution for (g) was dialysed only for three hours against the buffer at pH 12.0 with continuous stirring.

be regarded as a standard one, because its sample was prepared in the "buffered dialysate" of the microsomal supernatant, namely under most stable conditions (*cf.* our previous report (*4*) in detail). In Fig. 1 (a), the higher

peak (disignated as Component 2) represents the RNP, the lower one (Component 3*) being probably a partially dissociated RNP. When the sample is prepared in Miller-Golder's buffer of ionic strength 0.1 at pH 7.0, a small fast moving peak (Component 1) appears. Its mobility as well as an accompanying decrease in Component 2 followed by a compensatory increase in Component 3 suggests that Component 1 is RNA derived from the RNP by its dissociation.

In alkaline media the degradation of Component 2 proceeds further; the more alkaline the higher its degradation (Fig. 1 (c-e)) and it disappears almost completely at pH 12, only Component 1 and 3 of almost equal height being observed (Figs. 1 (f) and (g)). In Fig. 1 (g), the RNP solution was

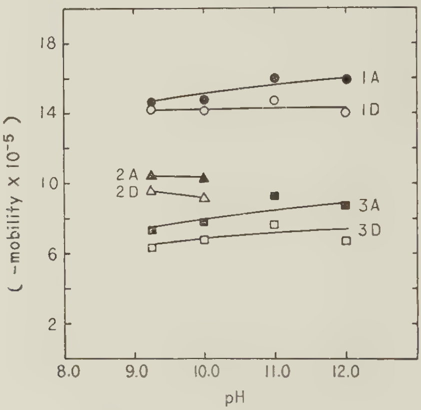

FIG. 2. Effect of alkali on the electrophoretic mobilities of the microsomal RNP.

Electrophoretic mobilities in 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} of each component of the RNP were calculated and plotted against pH. Each value represents the average of three or four determinations.

1 A : Component 1 ascending boundary, 1 D : Component 1 descending boundary, 2 A : Component 2 ascending boundary, 2 D : Compont 2 descending boundary, 3 A : Component 3 ascending boundary, 3 D : Component 3 descending boundary.

dialysed only for 3 hours against the Miller-Golder's buffer of pH 12 under continuous stirring because of unstability of RNA in alkaline media. Even under such a condition, the degradation of the RNP was complete as shown in Fig. 1 (g), while its Component 1 was more homogeneous than that in Fig. 1 (f).

* In this report, any component of the RNP of which mobility was smaller than that of the original RNP (Component 2) was designated as Component 3. It may be partially dissociated RNP (Component 2) or it's protein moiety.

In Fig. 2 are shown the average electrophoretic mobilities of each component in RNP solution in alkaline media. Fig. 1 (f) and (g) suggest that the Component 2, 1 and 3 of these figures represent the RNP, its nucleic acid and protein moiety respectively, the latter two being dissociated from RNP in alkaline buffer.

2. *Analysis of Component 1 and 3 Which Were Isolated Electrophoretically*—To ascertain whether or not the Component 1 and 3 in Fig. 1 (f) and (g) are RNA and a protein dissociated from the RNP respectively, their composition was analysed on both fractions isolated electrophoretically; after prolonged electrophoresis of the RNP solution (pH 12.0, ionic strength 0.1) in the cold, Component 1 and 3 were pipetted out from the ascending and descending limbs respectively and the amount of RNA and protein was determined by Littlefield's method. The results are summarized in Table I, which demonstrates that the RNP at pH 12 dissociates almost completely into RNA (Component 1) and protein (Component 3), amount of contamination in each component being only 3-13 per cent. A small amount of protein in Component 1 or of RNA in Component 3 may partialy be RNA-protein complexes, such as a nucleotide-amino acid complexes reported by Potter and Dounce (*8*), linked together by bondings other than electrostatic one.

TABLE I

RNA and the Protein Content of the Component 1 and 3

After prolonged electrophoresis of the RNP in the Miller-Golder's buffer of ionic strength 0.1 at pH 12.0, the component 1 and 3 were pipetted out of the ascending and descending limbs respectively, and the amount of RNA and protein were determined by the Schmidt-Thannhauser's method slightly modified by Littlefield et al.

Component	Component 1					Component 3				
Experiment No.	1	2	3	4	Mean	1	2	3	4	Mean
RNA (%)	94.3	96.6	86.8	91.4	92.3	13.2	6.1	2.6	3.2	6.3
Protein (%)	5.7	3.4	13.2	8.6	7.7	86.8	93.9	97.4	96.8	93.7

3. *Recombination of Dissociated RNA with Protein of the RNP in Neutral Media*—It is not only desirable for verifying the above-mentioned dissociation of the RNP into RNA and protein moiety, but also of special biological interest, to examine whether or not dissociated parts could recombine with each other under appropriate conditions. As has already been reported by one of us (*9*), the protein moiety of the RNP is hardly soluble in neutral or slightly acidic buffer, its minimum solubility being found at pH 5~6, while in acidic (pH 2~5) or neutral (pH 6~8) media it combines with yeast RNA.

Hence, recombination of RNA with protein was tested by returning pH of the RNP solution from 12.0 to 7.0. After confirming the complete dissociation of the RNP at pH 12.0 electrophoretically (Fig. 3 (a)), it was again dialysed against the Miller-Golder's buffer of pH 7.0 overnight in the cold (Fig. 3 (b)). Considerable increase in turbidity of solution accompanied with neutralization made it often difficult to perform electrophoretic analysis. As shown in Fig. 3, however, it was usually proved that neutralization

Ascending Descending

a)

b)

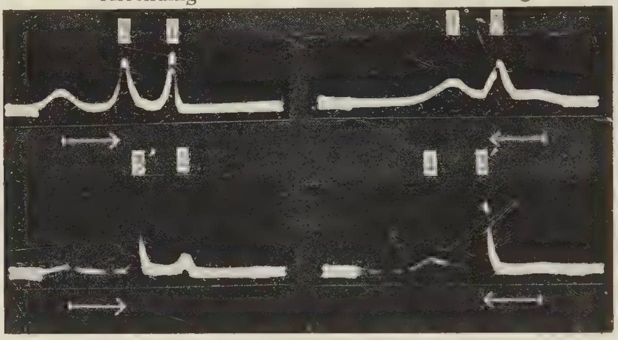

FIG. 3. Recombination of dissociated RNA and protein of the microsomal RNP.

Ascertaining the complete dissociation of the RNP at pH 12.0 (Fig. 3 (a)), the solution was reneutralized by the dialysis against the Miller-Golder's buffer of pH 7.0, and observed electrophoretically. Though the turbidity increase accompanied with the neutralization makes it often difficult to carry out electrophoresis, these figures show that the component 1 decreased remarkably suggesting that the component 3′ represents a recombined RNA-protein complex.

resulted in a remarkable diminution of Component 1 accompanied with a considerable increase in the slow Component (designated as Component 3' in this case). Mean values of ascending and descending mobility of Component 3' after neutralization were -9.6 and -8.2×10^{-5} cm^2. sec.$^{-1}$ volt^{-1} respectively (average of three samples), which were somewhat lower than the mobilities of Component 2 and nearly equal to those of Component 3 in original RNP solution at pH 7.0. These results seem to suggest that the protein moiety recombines with a part of RNA in neutral solution, the former giving a higher and more homogeneous pattern, though recombination did not recover fully its original mobility and dissociation would be regarded as irreversible one. Taking a fact into account that ovalbumine has shown hardly any interaction with yeast RNA in neutral media (*10*), it might be said at least that the protein of the RNP has a strong affinity to RNA to form a complex or complexes with it.

B. Effects of Saline on the Microsomal Ribonucleoprotein

1. Determination of Amount of RNA and Protein in the Sediments Fractionated by the Differential Centrifugation—Using the differential centrifugation, fractiona-

tion of the RNP in solutions of various NaCl concentrations was performed

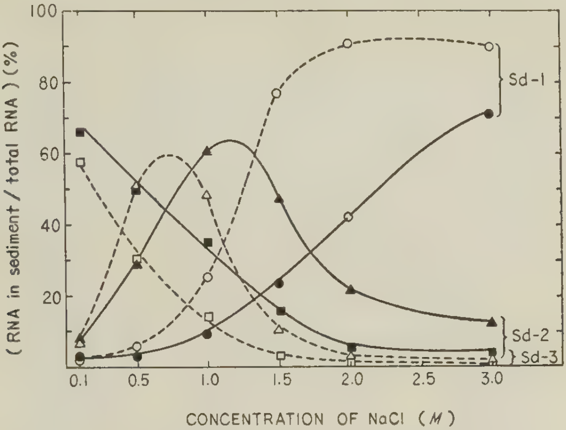

FIG. 4. Effect of saline on the microsomal RNP I.

Amount of RNA in each of the sediments 4 hours (continuous line) and 24 hours (broken line) after the addition of saline.

To the RNP solution, saline was added at final concentration of 0.1～3.0 M, and after a definite interval in the cold, the solution was centrifuged successively at $2{,}500 \times g$ for 20 minutes, $20{,}000 \times g$ for 20 minutes and $105{,}000 \times g$ for 120 minutes and the amount of RNA in each sediment such as Sd-1, Sd-2 and Sd-3 was determined. The values are per cent of RNA in any one sediment/total RNA and represent the average value of two parallel experiments.

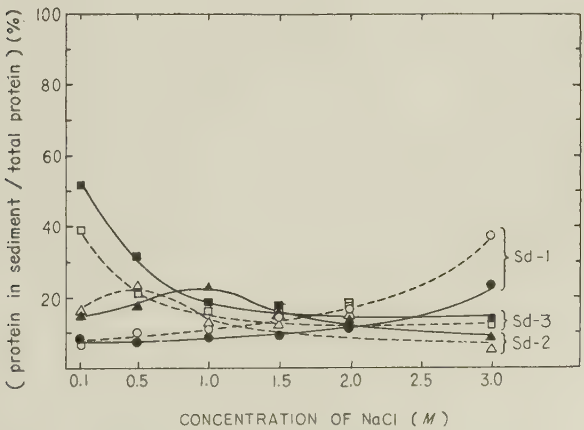

FIG. 5. Effect of saline on the microsomal RNP II.

Amount of protein in the sediments 4 hours (continuous) and 24 hours (broken line) after the addition of saline.

The values are per cent of protein in any one sediment/total protein and represent the average of two parallel experiments.

4 and 24 hours after addition of saline. The amount of RNA and protein as well as RNA content (RNA/RNA-protein) determined on each sediments are shown in Figs. 4, 5 and 6 respectively, all these values being averages of two experimental runs.

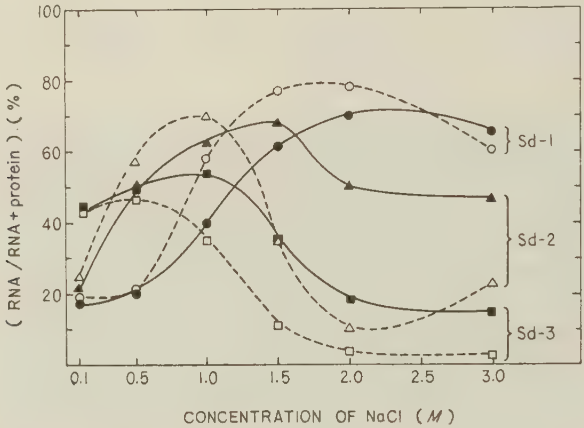

FIG. 6. Effect of saline on the RNA content of each sediment. Percentage RNA content (100 RNA/RNA+protein) was calculated from the values of the Figs. 4 and 5, and plotted against the concentration of saline.

When the strong saline was added to the RNP solution, its turbidity gradually increased, a fact which suggests the salting out of the RNP particles themselves and/or of their macromolecular components. As shown in Figs. 4 and 5, about 90 per cent of RNA was found in the Sd-1 in strong saline (2.0–3.0 *M*), while only a small amount of protein was recovered in this fraction. Accordingly, RNA content of this fraction in strong saline came up about 80 per cent as shown in Fig. 6, which is twice as much as that of the original RNA (about 40 per cent). Also it has recently reported that RNA, especially RNA of large molecular weight, is salted out in the strong saline (*11*, *12*). It is apparent, therefore, that it is mainly RNA that is salted out: The RNP is dissociated into RNA of considerably large molecular weight and protein in strong saline, the former being salted out by such a high concentration of NaCl (2$\sim$3 *M*).

Illustrations also show that such a dissociation effect of saline is probably effective even in lower concentration of 0.5–1.0 *M*. The amount of RNA and RNA/RNA+protein ratio of the Sd-2 attain their maximum value of 60 and 70 per cent respectively at about 1.0 *M*, beyond which concentration they decrease rapidly with remarkable augmentation of RNA in the Sd-1, while change in the amount of protein in the Sd-2 is only slight. These results suggest that RNA is dissociated even in saline of 0.5–1.0 *M*, which is salted out by more concentrated saline (2.0–3.0 *M*) and so found exclusively in the Sd-1.

2. *Salting out of RNA and Protein in 3.0 M Saline*—As shown in Fig. 6, RNA content of Sd-1 in 3.0 *M* saline was less than in 2.0 *M* and, at first glance, diminution of salting out of RNA seems to occur. But it is not the case, for amount of protein in the Sd-1 with 3 *M* saline is about twice as much as that with 2 *M* saline, while amount of RNA in both media is nearly equal. The following experiment clearly demonstrates it: the RNP solution was centrifuged at 2.500×*g* for 20 minutes in the cold, 1, 2, 4 and 24 hours after the addition of saline, its final concentration being 3.0 *M*, and

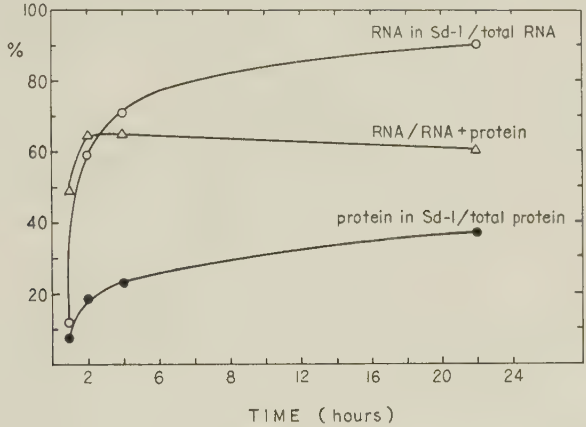

FIG. 7. Salting out of RNA and protein of the microsomal RNP in 3,0 *M* soline.

To the RNP, saline was added at a final concentratioh of 3.0 *M* and after a definite interval in the cold, the solutions were centrifuged at 2,500×*g* for 20 minutes and the amount of RNA (○) and protein (●) in each sediment were determined and RNA content (△) was calculated from these values. All the values represent the average of two experiments.

then amount of RNA and protein in the sediments were determined and their RNA content is calculated. The results illustrated in Fig. 7 show that the decrease in RNA content is not due to that in the amount of RNA salted out but due to the gradual increase of the protein sedimented, indicating that the protein moiety or the RNP itself is partially salted out in such a high concentration of salt as 3.0 *M* in the cold.

DISCUSSION

As the effects of acid on the microsomal RNP had already been reported (*9*), only the effects of alkali were observed by the electrophoretic method in the present study. The results mentioned above clearly demonstrated that, in alkaline buffer solution, the RNP was gradually dissociated into two components, a faster Component 1 and slower Component 3, and the former was almost exclusively composed of RNA and the latter was of protein,

when isolated electrophoretically at pH 12. Thence the dissociation process of the RNP seems to be represented by the following reaction as in the case of its autodegradation (*4*):

$$(n)\,\text{RNA}-\text{protein} \rightleftarrows (m)\,\text{RNA}+(n\text{-}m)\,\text{RNA}-\text{protein},$$

where (n) or (m) RNA means RNA is composed of n or m number of subunit-RNA.

The results are very similar to an electrophoretic study of calf thymus deoxyribonucleoprotein (DNP) carried out by Hemming and Jordan (*13*). However, it is to be noted that the dissociated RNA probably contains some amount of protein (or amino acid residues) in its molecule and that this dissociation is considered to be irreversible.

These results, however, do not always mean that the electrostatic force is mainly responsible for the bonding between RNA and protein in the RNP. RNA is unstable and easily degraded in alkali, so the dissociation of the RNP may be only a secondary effect of depolymerization of RNA. It is also possible that hydrogen bonded structure, probably involved in RNP molecule, may be disrupted by alkali as in the case of DNA (*14*).

The effects of saline on the RNP observed by the differential centrifugation, however, can be reasonably interpreted by the assumption that the RNP was dissociated into RNA and protein by adding salt and then RNA was selectively salted out in strong saline (2–3 *M*). Independent upon whether nucleic acid is RNA or DNA, therefore, the effect of neutral saline on the nucleoprotein seems to be the same in principle, its being chiefly due to disruption of electrostatic bonds, which is also responsible for the bonding of nucleic acid and protein moiety in RNP as in DNP.

It would be an interesting problem whether the dissociation of the RNP in strong saline is complete or not, or to what degree the RNP is dissociated, if incomplete. To our regret, however, it was impossible to obtain such a quantitative data, because protein or the RNP itself might be also somewhat salted out in strong saline and, moreover, all free RNA are not always salted out as proved by Kawade and Kitamura (*12*), its salting out in neutral saline being dependent not only upon salt concentration but also much upon its molecular weight and concentration.

One of the reasons why such an important characteristic of the RNP to dissociate into RNA and protein in strong saline in the cold has not been noted hitherto is probably due to the fact that RNA had been considered soluble in strong saline. It has been one of the popular method for the isolation of RNA to extract tissues with hot 10 per cent saline (*16–18*). The present finding suggests that the RNP is readily dissociated into RNA and protein by addition of 10 per cent saline in the cold, but RNA becomes extractable only when RNA is degraded to lower molecular one by heating at 60-100° over 60 minutes. Kerr and Seraidarian (*19*) have reported that RNA could be isolated by strong saline from beef pancreas RNP and suggested that its dissociation might be accomplished more rapidly and completely in the presence of salt of high concentration. Their RNA was soluble

in 3.0 *M* saline, however, and probably degraded one.

SUMMARY

The effects of alkaline buffer and neutral saline on the microsomal RNP isolated from the rat liver were examined by Tiselius' electrophoretic method and by differential centrifugation respectively. The results obtained are summarized as follow:

1. The RNP solutions were dialysed overnight against Miller-Golder's buffer of ionic strength 0.1 (pH 7.0–12.0) in the cold and their electrophoretic patterns were observed. The RNP (Component 2) is dissociated into two components (Component 1 and 3) in the alkaline buffer (pH 9.5–12), and the degradation was so enhanced at pH 12 that only two peaks of almost equal size (Component 1 and 3) were observed. Mobility of the former was nearly equal to that of free RNA.

2. Analysis of those Components 1 and 3 isolated by prolonged electrophoresis at pH 12 showed that the former was mainly composed of RNA and the latter was of protein in this case.

3. The dissociated RNP by dialysis at pH 12 was neutralized by redialysing against the same buffer at pH 7.0 overnight in the cold. The electrophoretic patterns obtained after neutralization revealed that Component 1 decreased remarkably and a slow Component (Component 3') showed compensatory increase, a fact which suggests that Component 3' represents a recombined RNA-protein complex.

4. To the RNP solutions saline was added in the cold, their final concentration being varied from 0.1 *M* to 3.0 *M*. Four and 24 hours after saline addition, these solutions were centrifuged at $2{,}500 \times g$, $20{,}000 \times g$ and $105{,}000 \times g$ successively. In strong saline (1.5–2.0 *M*), RNA content in the first sediment was about 80 per cent, that is twice as much as that in the original RNP (about 40 per cent) and the amount of RNA relative to total RNA found in it is nearly 90 per cent.

5. These values suggest that the RNP was dissociated into RNA and protein in strong saline and that RNA was selectively salted out in the cold. Even somewhat dilute saline (0.5–1.0 *M*) seems to be effective for the dissociation of the RNP.

6. In view of these results, it might be concluded that, besides hydrogen bonds, electrostatic bonds are also as important for the bonding between RNA and protein in the RNP as in the case of DNP.

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INTERACTION OF TAKA-AMYLASE A WITH SURFACE ACTIVE AGENT

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Studies on the interaction of surface active agent with protein throw light on the knowledge of protein structure. It is well known that surface active agent such as sodium dodecyl sulfate (SDS) is a strong denaturant for proteins. A relatively small quantity of surfactant is sufficient to denature protein although a large quantity of urea is needed for the denaturation of the same amount of protein. Moreover, the properties of proteins are considerably modified in the presence of surface active agent. Accordingly, the interaction of surface active agent with protein draws nowadays an increasing attention of many investigators (*1*).

Hitherto, the studies were made mainly with proteins such as serum albumin and egg albumin (*2–11*). The effect of surface active agents on enzyme is a fascinating problem and has been studied only from the standpoint of its enzyme activity (*12–18*, *25*). On the other hand, amylase, an enzyme protein, is practically used for desizing the textile without inactivation even in the presence of some surface active agents. Amylase may be assumed to be a resistant protein against the action of surface active agent. In this connection, we chose Taka-amylase A (TAA, α-amylase of *Aspergillus oryzae*) as an example of protein and the interaction of SDS with this protein was studied electrophoretically. The results obtained were analysed in terms of chemical kinetics. In consequence, Taka-amylase A was found to be very stable in comparison with usual globular proteins such as serum albumin.

EXPERIMENTALS

Taka-amylase A—Taka-amylase A was prepared by the procedure according to Akabori and his collaborators (*19*) from "Takadiastase Sankyo" and recrystallized three times from aqueous acetone. TAA was dissolved in 0.1 *M* sodium acetate buffer at pH 5.0 in concentration 3.75 per cent and stored in a refrigerator. As this sample was dialyzed against 0.1 *M* sodium acetate buffer to remove acetone, calcium ions which strongly protect TAA molecule from denaturation seemed to be removed excepting one mole per mole TAA.

Sodium Dodecyl Sulfate—Dodecyl alcohol of high purity which was prepared by reduction of molecularly distilled lauric acid was sulfated by Dreger's method (*20*). It was recrystallized from butanol saturated with water and extracted with ether to remove the unreacted lauryl alcohol.

Buffer Solution—Experiments were all carried out in sodium acetate buffer, 0.1 *M* ($\Gamma/2=0.068$) at pH 5.0. It was confirmed by a glass electrode pH-meter that no change of

of pH of the solution was caused by the addition of SDS, within the range of concentration of SDS used in the present experiment.

Prior to electrophoresis, the solutions of TAA mixed with SDS solution of a definite concentration were dialysed immediately after the mixing for various intervals of time against a large volume of SDS-buffer mixture at the same concentration. The dialysing apparatus was frequently shaken to certify the same concentration of SDS in the protein solution and that of the outer dialysate.

After a definite time of interaction, electrophoresis was carried out with these solutions, making a boundary between the protein solution and the equilibration dialysate being layered over the protein solution in the cell. The dialysis was carried out at 20°, 25°, or 30°. Electrophoresis was performed at 20±0.05°C by a moving boundary method using Hitachi's electrophoresis apparatus, Model HT-B equipped with the schlieren diagonal system. The voltage gradient was in the range of 3.9–4.5 volts/cm.

The concentration of TAA was determined by the micro-Kjeldahl method assuming the nitrogen content of TAA to be 15.0 per cent (*19*).

RESULTS

Effect of the Time of Interaction—As shown in Fig. 1, the protein migrated to anode and two distinctly separated peaks appeared when electrophoresis

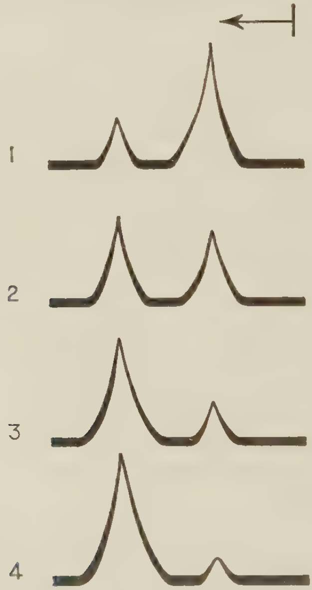

FIG. 1. Electrophoretic patterns (descending limb) of native (slower peaks) and SDS-denatured (faster peaks) TAA reacted with 0.01 *M* SDS at 20°. TAA: 0.38 per cent. Reaction time. 1, 12 hours; 2, 50 hours; 3, 74 hours; 4, 100 hours. Electrophoresis time: about 20 minutes.

was conducted with the SDS treated amylase solution. Because of the low velocity of denaturation of TAA by SDS it was found no sign of disturbance

at the boundary in the course of electrophoresis even after some twenty minutes. The longer the reaction time before electrophoresis was, the more the portion of the fast migrating component, and the less that of the slow migrating component was. Among these two portions the one that migrates with low mobility would be identified with the native TAA, by comparing the mobility with that of the native TAA in the absence of SDS. On the other hand, the fast moving component is attributed to the denatured TAA which increases its negative charge by combining with many SDS anions.

Effect of Concentration of SDS—Next, the effect of concentration of added SDS was examined. The mobilities found in these experiments are shown in Table I. The mobility of the slower component was nearly constant

TABLE I

Effect of Concentration of SDS on the Mobility of TAA

(Descending boundary)

Cocentration of SDS (M)	Mobility ($cm^2/sec./volt \times 10^4$)	
	Slow moving peak	Fast moving peak
0	0.902	
1.00×10^{-3}	0.908	1.30
1.00×10^{-2}	0.902	2.12
2.00×10^{-2}	0.880	2.04
3.00×10^{-2}	0.804	1.76

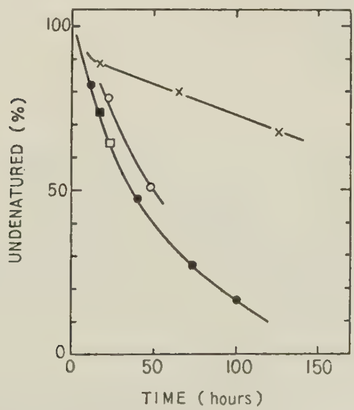

FIG. 2. Effect of the concentration of SDS on the rate of denaturation of TAA by SDS at 20° —×—, 1.00×10^{-3} M; —○—, 5.00×10^{-3} M; —●—, 1.00×10^{-2} M; —□—, 2.00×10^{-2} M; —■—, 4.00×10^{-2} M SDS.

irrespective of the amount of added SDS even though the mobility decreases to some extent when the large excess of SDS was added to the protein

solution. The faster denatured component increased first its mobility with the concentration of SDS but reached nearly constant value when the concentration of SDS exceeded a certain limit.

By measuring the area under separate peaks in the electrophoretic pattern, the concentration ratio of the denatured to the native TAA can be estimated. The rate of denaturation might be determined if the ratio was determined as a function of dialysing time, namely, time of interaction. The effect of the concentration of SDS upon the rate of denaturation was also examined. The results obtained are shown in Fig. 2. It is very interesting that the rate of denaturation is independent of SDS concentration if it exceeds a certain concentration. This concentration corresponds to the critical micelle concentration of the surfactant.

Effect of Temperature—The effect of dialysing temperature, namely reaction temperature was also investigated and the results are shown in Fig. 3. In Fig. 4, log (undenatured percentage) was plotted against the reaction period. Linear relation was observed between log (undenatured percentage) and the interaction time. Accordingly, denaturation of TAA by the action of SDS obeys the first order kinetics as those of many other proteins.

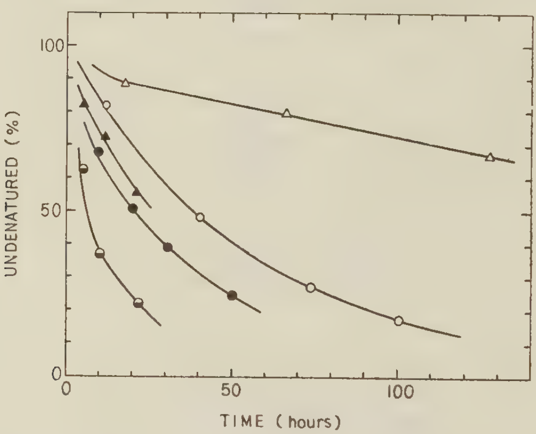

FIG. 3. Effect of temperature on the rate of denaturation of TAA by SDS. —△—, 1.00×10^{-3} *M* SDS 20°; —▲—, 1.00×10^{-3} *M* SDS at 30°; —○—, 1.00×10^{-2} *M* SDS at 20°; —●—, 1.00×10^{-2} *M* SDS at 25° and —◒—, 1.00×10^{-2} *M* SDS at 30°.

Velocity Constant and Other Parameters of Denaturation Kinetics—The velocity constant for this denaturation was calculated from the slope of the straight lines given in Fig. 4. The results are shown in Table II. From the variation of velocity constant with temperature, activation energy for denaturation of this protein was calculated according to A r r h e n i u s' equation. Further, free energy, enthalpy and entropy of activation for denaturation were calculated by means of E y r i n g's equation (*21*). The results are listed in Table III.

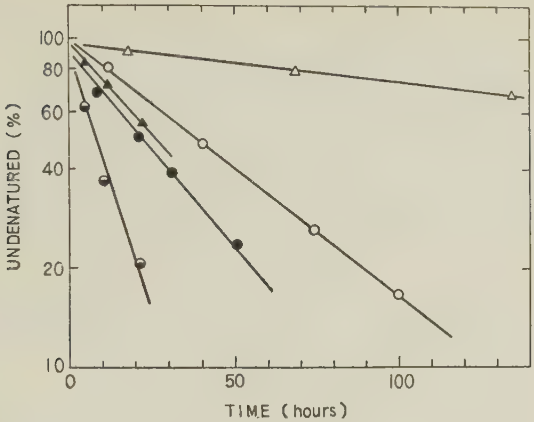

FIG. 4. Plot of log (undenatured percentage) *versus* time. All symbols are the same with those in Fig. 3.

TABLE II

Specific Rate of Denaturation of TAA by SDS

Concentration of SDS (M)	Temperature $(°C)$	Specific reaction rate, k (sec^{-1})
0.001	20	9.28×10^{-7}
0.001	30	8.06×10^{-6}
0.01	20	5.00×10^{-6}
0.01	25	7.16×10^{-6}
0.01	30	2.18×10^{-5}

TABLE III

Free Energy (ΔF^{++}), *Enthalpy* (ΔH^{++}) *and Entropy* (ΔS^{++}) *of Activation for Denaturation*

Concentration of SDS (M)	ΔF^{++} (kcal/mole)	ΔH^{++} (kcal/mole)	ΔS^{++} (e.u./mole)
0.001	25.2	38.4	45.1
0.01	24.2	25.0	2.7

DISCUSSION

Globular proteins such as serum albumin are readily denatured by sodium dodecyl sulfate. According to Briggs and his collaborators (*5*, *6*), denaturation of protein by the action of surface active agent is caused by the combination of five to ten surfactant ions per protein molecule which

serve like a wedge to destroy the protein structure. Only if there exists an insufficient amount of surfactant ions to cause such destruction the peak for native protein appears in electrophoretic patterns besides that of denatured protein. The mobility of native serum albumin increases as a function of concentration of surfactant ions because of the adsorption of surface active agents. In Table IV the mobilities of undenatured bovine serum albumin in the presence of small amount of SDS which were cited from the paper by Briggs and his collaborators (*5*) how the considerable effect of concentra-

TABLE IV

Effect of Concentration of SDS on the Mobility of Slow Moving Peak of Bovine Serum Albumin (*5*)

(Descending boundary)

Concentration of SDS ($M \times 10^5$)	Mobility ($cm^2/sec./volt \times 10^5$)
0	6.15
1.59	6.36
4.60	7.36
16.1	8.20
48.5	8.72
80.2	11.00

tion of SDS. On the other hand, the mobility of native Taka-amylase A is independent of the concentration of surfactant ions, if the concentration is less than $1.00 \times 10^{-2}\ M$ as mentioned above. When the concentration of the surfactant exceeds $2 \times 10^{-2}\ M$, the mobility of the protein decreases rather than increases. This decrease of the mobility might be caused by the considerable increase of ionic strength of the medium. From these experimental facts, SDS ions seem to be hardly adsorbed on TAA molecule. The reason why TAA can hardly adsorb SDS ions may be partly due to lack of adsorbing sites for SDS ions on the surface of protein molecule and partly to the compact rigid structure of this enzyme as previously reported by one of the present authors (*22*, *23*). The interacting sites such as ammonium and imidazolium groups are presumably buried in the molecule or masked by intramolecular bonding so as not accessible to SDS ion. Conversely, the difficulty of denaturation of TAA by SDS may support our inference that the molecule of TAA should be rigid and compact.

By analysing the electrophoretic patterns, it was found that the rate of denaturation of TAA was independent of the concentration of SDS if it exceeded a certain concentration which corresponds practically to the critical micelle concentration of the surfactant. From this fact, it seems that not the micelles of this surfactant but single ions should cause the denaturation of TAA.

The entropy of activation of denaturation for the system to which SDS

was added in a concentration of 0.01 M was 2.7 e.u./mole and far less than for the system to which SDS was added in a concentration of 0.001 M, *i.e.* 45.1 e.u./mole. This fact might have some concern with the fact that SDS binds with the protein as if it forms micelle on the surface of protein when the concentration of SDS exceeds the critical micelle concentration of the surfactant (*24*).

The value of free energy of activation of denaturation, ΔF^{++} 24.5 or 25.2 kcal/mole lies in the range of 23～28 kcal/mole which is a generally accepted range of free energy of activation of denaturation.

Most proteins hitherto studied on their interaction with surface active agent are easily available ones such as egg albumin or serum albumin. These proteins are readily denatured by surface active agents. Consequently, it is generally accepted that the surface active agent is a powerful denaturant for proteins and denatures protein with ease. However, it is noteworthy that there is a resistant protein against surfactant such as TAA. Ikemiya (*25*) who investigated inhibition of various amylases by surface active agents found that TAA is especially resistant to the action of SDS down to pH 4.2 in good agreement with our results. The reason why TAA is difficult to be denatured by the action of surface active agent might be as follows. Proteins which are easily denatured, *e.g.* serum albumin, combine with considerable amount of surfactant ions even in an undenatured state. The protein molecules suffer remarkable distortion which eventually leads to the denaturation of protein. On the other hand, TAA as mentioned above, combine scarcely with SDS ions in a native state. Accordingly, SDS would not affect as a direct denaturant but act as an indirect agent which causes to reduce the activation energy of thermal denaturation. Enzyme activity of TAA is markedly inhibited in the solution of which pH is below 4.0. This inhibition of TAA might not be due to primary effect of the action of SDS, because TAA is easily acid-denatured even in the absence of SDS as shown by Oikawa (*26*).

SUMMARY

1. The interaction of Taka-amylase A with sodium dodecyl sulfate was investigated electrophoretically.
2. In a solution containing SDS, the mobility of native TAA is almost independent of SDS concentration of the medium, suggesting meager interaction of SDS with TAA.
3. TAA is denatured very slowly by SDS, contrary to the very rapid denaturation of most other proteins by SDS.
4. The denaturation of TAA in the presence of SDS in the solution was examined kinetically by analysing electrophoretic patterns. Not micelles but single ions of SDS may be responsible for the denaturation of TAA.
5. Plausible mechanism of denaturation of TAA by SDS was suggested.

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A PROTEOLYTIC ENZYME OF STREPTOMYCES GRISEUS

IV. GENERAL PROPERTIES OF STREPTOMYCES GRISEUS PROTEASE*

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(Received for publication, June 9, 1959)

As previously reported (*1*, *2*, *3*), a protease of *Streptomyces griseus* was obtained as a highly purified, homogeneous enzyme preparation. Some investigations on the general properties of this enzyme have been conducted by the use of the preparation and the results obtained are presented in this paper.

MATERIALS AND METHODS

Enzyme—A highly purified, lyophilized enzyme preparation described in the preceding paper (*3*) was mainly used throughout the present work. The homogeneity of this preparation was previously confirmed by ultracentrifugal, electrophoretic, and enzymological analyses (*3*).

Assay for Casein Digestion Activity (*Proteinase Activity*)—The enzyme activity for digesting casein was determined by the casein-Folin color method (*1*) and the activity unit was represented by the *PU* value (*1*). For the assay of the activity at various pH, suitable buffers** were used for preparing the enzyme solution instead of the usual phosphate buffer (pH 7.4) and the pH of the casein solution was adjusted at the corresponding pH with acetic acid or sodium hydroxide.

Assay for Peptide Bond Hydrolysing Activity (*Peptidase Activity*)—The casein-formol titration method was used for the present purpose. One ml. of the enzyme solution suitable diluted with 0.01 M calcium acetate solution and containing about 2×10^{-2} *PU* was added to 5 ml. of 3 per cent casein solution (pH 7.4) and the mixture was kept at 30° for 20 minutes. After reaction, 5 ml. of 35 per cent formol solution was added to the mixture and electrometric titration was carried out with 0.02 N sodium hydroxide. The end point of the titration was pH 8.5. The titration values were corrected by subtracting the values of blanks which were prepared by mixing substrate solution with the formol solution followed by addition of the enzyme.

For the assay of the activity at various pH, the pH of the casein solution was previously adjusted at assigned values of pH by acetic acid or sodium hydroxide. Alteration of the pH caused by the addition of enzyme solution was in every case within 0.1.

* A part of the data was reported at Symposium on Enzyme Chemistry at the University of Tokyo in July 1956 and most parts of the work were published in Japanese in the *Reports of the Institute of Physical and Chemical Research*, **35**, 84 (1959).

** A $M/15$ sodium phosphate buffer system was used in the pH range of 5.5-8.0 and $M/10$ sodium borate buffer system in the pH range of 8.0–10.0.

RESULTS AND DISCUSSION

Shape of Crystalline Enzyme—As previously reported (*1*, *2*), *St. griseus* protease is obtainable as small needle crystals and the specific activity of this preparation is about 8–12 $\times 10^{-2}$ *PU*/mg.-N. The preparation of this enzyme as the crystalline form, however, was fairly difficult and still somewhat defective, but the highly purified preparation was of fair homogeneity which was confirmed by several analyses (*3*).

Molecular Weight—As described in the preceding paper (*3*), the sedimentation constant of the enzyme was determined as $s_{20,\,w}=2.8\times10^{-13}$ (cm./sec.)/(dyne/g.) by ultracentrifugal analysis.

Diffusion measurements were carried out at 20° in a Tiselius apparatus used for the electrophoresis experiments. A 3 per cent enzyme solution in 0.1 *M* sodium acetate used. From the data obtained, the values of the diffusion constant were computed to be $D_{A\,20,\,w}=13.1\times10^{-7}$ (cm.^{2}/sec.)* and $D_{\mu\,20,\,w}=13.4\times10^{-7}$ (cm.^{2}/sec.)*.

Taking $s=2.8\times10^{-13}$, $D=13.1\times10^{-7}$ and $V_t=V_{20}=0.74$ (V is the specific volume), the molecular weight of the protease according to the expression $M_{SD}=RT_s/D\ (1\text{-}V\rho)$ becomes $M_{SD}=20{,}000$.

Isoelectric Point—Experiments were carried out in a Tiselius apparatus as

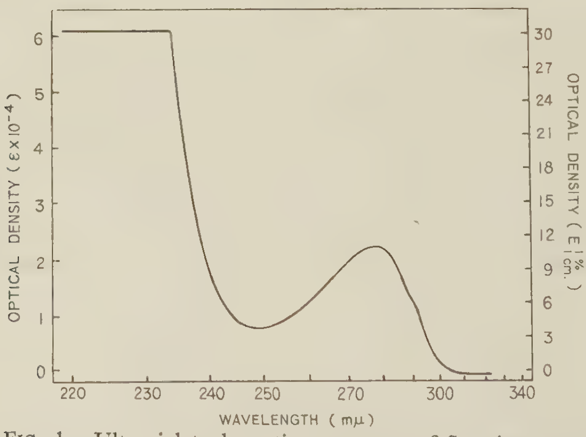

FIG. 1. Ultraviolet absorption spectrum of *St. griseus* protease.
Apparatus: A Beckman-type (model DK-2) spectrophotometer.
Solvent: 0.1 *M* sodium acetate solution, pH 6.5.

previously reported (*3*). The results obtained indicate that the isoelectric

* D_A is the diffusion constant calculated from diffusion diagram by the Maximum ordinate-area method. D_μ is also the diffusion constant calculated from the same diagram by the Maximum ordinate method. The fair agreement with each other of the two values which were calculated from the same diagram by two different methods proves the symmetry of the diagram and accordingly the homogeneity of the diffusing substance.

point of the protease is presumed to be at pH 5.0–5.5. Further detailed experiments, however, are needed on this subject to determine more exact value.

Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of the protease was obtained by the use of a Beckman-type (model DK-2) spectrophotometer. As shown in Fig. 1, the spectrum of this enzyme is of the pattern of a typical protein with the maximum absorption at 280 mμ indicating the presence of tyrosine residue in the enzyme, while another small rise at 290 mμ suggesting the presence of tryptophane residue. The molecular extinction coefficient of the protease was computed to be $\varepsilon = 2.2 \times 10^4$ at 280 mμ.

Solubility—*St. griseus* protease is easily soluble in distilled water or diluted salts solution. The enzyme is soluble in ammonium sulfate solution of up to 0.3 saturation (w/v), but at 0.4 saturation (w/v) it becomes almost insoluble. At ice cold temperature, the enzyme is soluble in acetone solution of 50 per cent concentration but not of 60 per cent. The solubility of the enzyme in alcohols is somewhat larger than in acetone.

Element Composition—The composing elements of the highly purified preparation are as follows: C 52.0, H 6.8, N 14.8, S trace, and ash 0.8 per cent. Almost every metal found in the above is calcium.

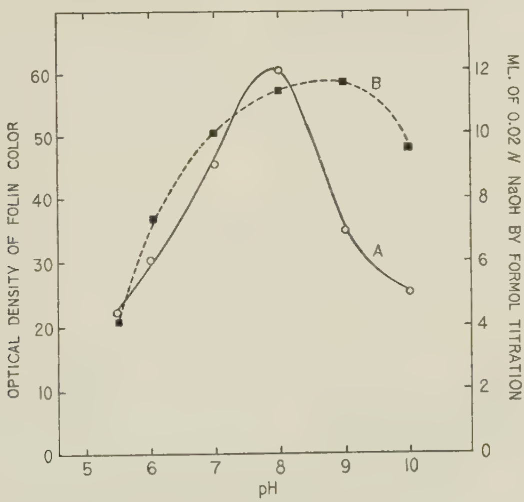

FIG. 2. Relation between pH and enzyme activity of *St. griseus* protease.

(A): The pH-proteinase activity curve determined by the casein-Folin color method.

(B): The pH-peptidase activity curve determined by the casein-formol titration method.

Enzyme Activity and pH—Relation between pH and enzyme activity of *St. griseus* protease determined by the casein-Folin color method and casein-formol

titration method is shown in Fig. 2. The enzyme showed the maximum proteinase activity at pH 8.0 or so and the maximum peptidase activity at the pH range of 8–9.

Stability—The enzyme was very unstable in dialysis against distilled water and a marked reduction of enzyme activity was observed in 24 hours' dialysis even at low temperature. Such inactivation, however, was completely prevented by the addition of small amount of calcium ion to the outside distilled water from which we learn that calcium ion is highly effective in preventing the enzyme activity. The activity of its diluted solution containing calcium ion was considerably stable at neutral pH in a refrigerator. The other inorganic ions, excepting strontium, had no protective effect on this protease. Further detailed report on this subject will be presented in the next paper.

Relation between stability of the enzyme and pH of the solution is shown in Fig. 3 which shows that the enzyme was highly stable at the pH range of 6.0–9.0, but fairly unstable below pH 4 and above pH 10. In these experiments, the enzyme preparation was dissolved in suitable buffer solutions containing 0.005 *M* calcium ion and kept at various pH at room temperature. The residual activity of the enzyme was determined by the casein-Folin color method after the solutions were neutrized by addition of acid or base.

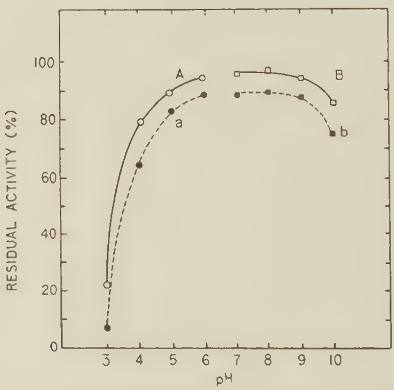

FIG. 3. Relation between pH and stability of *St. griseus* protease.
(A): After stored for 5 hours in McIlvaine buffer.
(a): After stored for 24 hours in McIlvaine buffer.
(B): After stored for 5 hours in sodium borate buffer.
(b): After stored for 24 hours in sodium borate buffer.

Even in the state of diluted solution, the enzyme was fairly stable for heating (Fig. 4—curve A). The enzyme solution, which was made up in distilled water, was kept at 40–100° for 10 minutes and the residual activity was measured by the casein-Folin color method. The stability of the enzyme in heating was found very much increased by the coexistence of substrate, about 70 per cent of the initial activity still remained after 10

minutes' heating at 60° (Fig. 4—curve B).

On the other hand, as the velocity of chemical reaction becomes larger in proportion to the reaction temperature, the relation of the activity to the

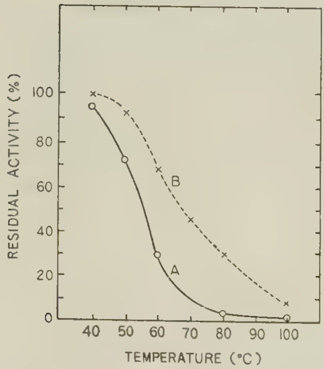

FIG. 4. Relation between temperature and stability of *St. griseus* protease.

(A): After heating the enzyme in distilled water for 10 minutes.

(B): After heating the enzyme in 1 per cent casein solution for 10 minutes.

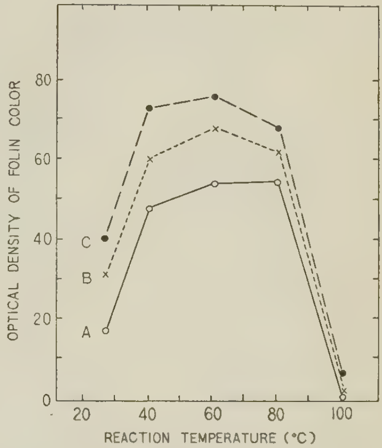

FIG. 5. Relation between reaction temperature and enzyme activity of *St. griseus* protease. (A): By 15 minutes reaction. (B): By 30 minutes reaction. (C): By 45 minutes reaction. Experiments were carried out by adding 1ml. of enzyme solution to 1 ml. of 2 per cent casein solution at pH 7.4 at various temperatures. Measurements of the amount of resulting digestion products were performed by the usual casein-Folin color method (*1*).

reaction temperature was observed. The enzyme showed the maximum proteolytic activity at 80° in 15 minutes reaction and at 60° in 30–45 minutes reaction (Fig. 5). The so-called optimum temperature for this enzyme seems to be 40–60°.

Hydrolysis of Various Proteins—St. griseus protease was found to digest almost all kinds of proteins such as casein, human serum albumin, bovine serum albumin, ovalbumin, human serum γ-globulin, hemoglobin, fibrinogen, gelatin, edestin, soybean glycinin, wheat gluten (glutenin), rice orizenin, *etc.* The scleroproteins such as keratin and silk firbroin were hardly digested by this enzyme.

A protein denatured by heating or urea treatment was hydrolyzed by this enzyme faster than the untreated. The latter, however, was hydrolyzed fairly well if the reaction was prolonged. Muscular and dermal tissues of a living mouse were found completely liquefied and fallen off by intramuscular injection of the enzyme. The results obtained are summarized in Table I.

All the denatured proteins tested were digested by this protease till the proteins caused no precipitation by 0.4 M trichloroacetic acid. This property is not observed in the cases of other neutral proteinases such as trypsin and

chymotrypsin, in which some of the digested products are found to remain still insoluble in 0.4 *M* trichloroacetic acid solution. The large extent of

TABLE I

Hydrolysis of Various Proteins by St. griseus Protease

Substrate	Relative velocity of hydrolysis		
	Protein untreated	Denatured protein by urea treatment[2]	Denatured protein by heat treatment[3]
Human serum albumin	0.11	0.18	0.18[4]
Ovalbumin	0.06	0.21	0.18[4]
Human serum γ-globulin	0.06	0.27	0.19[4]
Fibrinogen	0.31	—	0.54[4]
Hemoglobin	0.49	0.64	0.53[4]
Casein	—	—	0.63
Edestin	0.09	0.42	0.54

1) Relative velocity of hydrolysis was measured by the casein-Folin color method, in which 0.4 *M* trichloroacetic acid (TCA) solution was used instead of the usual 0.1 *M* TCA solution. The numerical figures in the Table indicate the optical density of the resulting Folin color.

2) These proteins were denatured by mixing with urea (50 g./100 ml.) and kept at room temperature for 24 hours. After treatment, the coexisting urea was removed by 48 hours dialysis. The denatured proteins were diluted to 2 per cent concentration.

3) These proteins were denatured by heating at 100° for 10 minutes in solution of 2 per cent concentration.

4) These proteins coagulate by heating.

hydrolysis of proteins by *St. griseus* protease seems to be due to the wide specificity of this protease.

SUMMARY

A study was made on several properties of *St. griseus* protease obtained as a highly purified, homogeneous enzyme preparation.

1. The crystalline form is of small colorless needles. The molecular weight is about 20,000, which was calculated from the sedimentation constant ($s_{20,w}=2.8\ S$) and also from the diffusion constant ($D_{A\ 20,w}=13.1\times10^{-7}$, $D_{\mu\ 20,w}=13.4\times10^{-7}$ cm.^{2}/sec.). Ultraviolet absorption spectrum of *St. griseus* protease is of the type characteristic of most proteins and shows no indication of the presence of a special group.

2. The optimum pH for the enzyme activity measured by the casein-Folin color method is about 8.0 and that by the casein-formol titration method is 8–9.

3. The enzyme is fairly stable at pH 6–9 below 40° but only slightly stable above 60°. The stability in heating of this enzyme becomes much better in the presence of substrates. Calcium ion is an essential factor for stabilizing this protease.

4. This protease is capable of digesting almost all kinds of proteins, excepting the insoluble scleroproteins. The velocity of hydrolysis for denatured proteins is much higher than that for the native one.

The authors wish to express their thanks to Dr. T. Akahira, Dr. M. Yanagita, Prof. K. Sakaguchi, Prof. K. Okunuki and Dr. B. Hagihara for their kind guidance and useful suggestions. The authors are also grateful to Mr. S. Fujita for preparation of enzyme, to Mrs. N. Igarashi for her helpful assistance.

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NOTES

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CHROMATOGRAPHIC SEPARATION OF PHENOLIC ACIDS

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(Received for publication, April 9, 1959)

Chromatographic separation of phenolic acids has been carried out mainly by ion exchange and paper chromatographic methods (*1–5*). Although these methods afford excellent resolving power, their applications to the quantitative determination and large-scaled separation of phenolic acids are accompanied with some difficulties. In order to remove these difficulties, the employment of cation exchange resins as adsorbents was investigated. These resins have been used for the separation of flavonoids (*6*), derivatives of phenol (*7*), DNP-amines (*8*) and lower fatty acids (*9*). We have succeeded in separating some representatives of the phenolic acids, *e.g.* gentisic, homogentisic, protocatechuic and homoprotocatechuic acid.

Since the pK of the phenolic acids varies from 1.3 to 4.5, it was necessary to use an acidic solvent (solvent A or B as shown in Table I) to suppress the ionization of the carboxyl group in both solute and adsorbent when a carboxylic acid type cation exchange resin was used. When a sulfonic acid type resin was used, the pH of the eluent was chosen at pH 4.34 ± 0.02 in order to reduce the adsorption and to facilitate the separation of acids differing in pK value (solvent C in Table I).

EXPERIMENTAL

Phenolic Acids—The homogentisic acid used was isolated from the urine of an alcaptonuric. Homoprotocatechuic acid was kindly supplied by Dr. K. Kunita and *p*-hydroxyphenylpropionic acid by Dr. K. Oshima. Ortho- and meta-hydroxyphenylacetic acid was a preparation synthesized by Mr. S. Nakanishi. Other acids were commercial products.

Organic Solvents—Methylethylketone, acetone and ethanol (99 per cent) were distilled before use. Sodium acetate was for analytical use. The composition of the solvents used for chromatography is shown in Table I.

Ion Exchange Resins—Amberlite IRC 50 (A. G.) and Duolite C 25* were used. The

* In the earlier stage of this investigation, Amberlite IR 112 was used. Since this resin is now obsolete, we abandoned its use and substituted it by Duolite C 25, a sulfonic acid type resin with an amorphous polystyrene matrix.

former was pulverized and washed as previously described (*9*). The latter was converted into the sodium ion form and pulverized in a ball mill. The pulverized resin was suspended in water, passed through a 200 mesh screen with running water and then filtered through a 300 mesh screen in order to remove fine particles. The filtered resin was washed according to the method of W. H. Stein *et al.* (*10*).

TABLE I
Composition of the Solvents

Solvent	Composition
A	Methylethylketone : acetone : 0.2 N hydrochloric acid *2 : 1 : 6
B	Methylethylketone : acetone : 0.2 N hydrochloric acid *2 : 1 : 9
C	Twenty one grams of citric acid monohydrate and six g. of sodium hydroxide were dissolved in distilled water and diluted to 1 liter, then mixed with 250 *ml.* of 99 per cent ethanol**.

* Mixed by volume.
** Buffer solvents containing ethanol were used previously by Hirs *et al.* (*11*) and Drèze and Reith (*12*).

Chromatographic Column—Details of the chromatographic tube were described previously (*9*). The ion excnange resin suspended in the solvent to be used for chromatography was poured through a funnel into the chromatographic tube and allowed to settle. The size of the columns were 0.76×95 cm. and 0.76×112 cm. for Amberlite IRC 50 and 0.8×95 cm. for Duolite C 25.

Chromatographic Separation and Quantitative Analysis of Synthetic Mixtures—One ml. of a solution of phenolic acids in the same solvent as was used for packing of the column was added on the column and elution was performed with the same solvent. The effluent was collected in fractions of 20 or 40 drops in test tubes with graduation mark at 3.5 ml. by using a drop count type automatic fraction collector.

The quantitative analysis was performed spectrophotometrically. When acidic solvents were used, 1.0 ml. of $N/10$ sodium acetate solution was added to each of those phenolic acid fractions which contained 40 drops of the effluent, 1.0 ml. of $N/20$ sodium acetate solution was added to the fractions containining 20 drops of the effluent and 1.0 ml. of $N/2$ sodium acetate solution was added to the salicylic acid and non-hydroxylated acid fractions. The test tubes containing the fractions were placed in a suitable rack and heated in a boiling water bath for about one hour and a half to remove acetone and methylethylketone. After the organic solvents were evaporated off, an acidic diluent, prepared by mixing 10 ml. of conc. hydrochloric acid, 100 ml. of N sodium acetate solution and 90 ml. of water, was added to the fractions containing dihydroxy compounds, and $N/2$ sodium acetate solution was added to the monohydroxy and non-hydroxylated acid fractions to make each fraction up to a total volume of 3.5 ml. The fractions containing gentisic acid were diluted (to give 3.5 ml.) with the acidic diluent without removal of

organic solvent, since the solution turned yellow on heating in a water bath. When solvent C was used, the fractions containing dihydroxy acid (with the exception of the fraction containing protocatechuic acid) were diluted (to give 3.5 ml.) with the same solvent as was used for the elution, but other fractions were diluted with $N/2$ sodium acetate solution (to give 3.5 ml.). The absorption was then measured at the wave lengths shown in Table II using a Beckman model DU quartz spectrophotometer. Amberlite IRC 50 resin could be used rpeatedly, since the resin was used with an acid solvent. Column of Duolite C 25 may be washed with $N/5$ sodium hydroxide solution containing 20 per cent ethanol to remove strongly adsorbed substances.

TABLE II

Recovery of Phenolic Acids from the Chromatographic Column

Phenolic acid	No. of acid in the Fig.	Wave length used for the assay ($m\mu$)	Recovery (*per cent*)		
			A*	B*	C*
Homogentisic acid	1	290		65	98
Homoprotocatechuic acid	2	280		99	80
Protocatechuic acid	3	260		93	101
p-Hydroxyphenylacetic acid	4	280	96	93	98
m-Hydroxyphenylacetic acid	5	280	89	92	83
o-Hydroxyphenylacetic acid	6	280		98	83
Gentisic acid	7	330	98		94
p-Hydroxybenzoic acid	8	260	90	94	95
m-Hydroxybenzoic acid	9	280	108	104	90
Salicylic acid	10	295	99		99
p-Hydroxyphenylpropionic acid	11	280	109	100	85
Phenylacetic acid	12	260	96		
Benzoic acid	13	260	97		
β-Phenylpropionic acid	14	260	101		

* Solvent used for the chromatographic separation.

RESULTS AND DISCUSSION

As shown in Figs. 1 to 3, the separation of phenolic acids was performed in three different solvent systems. The resolution was very sensitive to the slight changes in the ambient temperature, so that it was necessary to keep the column at constant temperature by wrapping it with a jacket (*10*). The capacity of the column was large enough and about 30 μM of each acid could be added, although the optimum load was 1 to 5 μM of each acid.

The elution sequence of each acid was mainly governed by its polarity and molecular weight, because the resin phase acted as non-polar adsorbent. In some cases reversion of the elution sequence and overlapping of acids of

different polarity and molecular weight was observed, suggesting that the position of the hydroxyl group relative to the carboxyl group and side chain may play an important role in the partition of solute between resin and liquid phase.

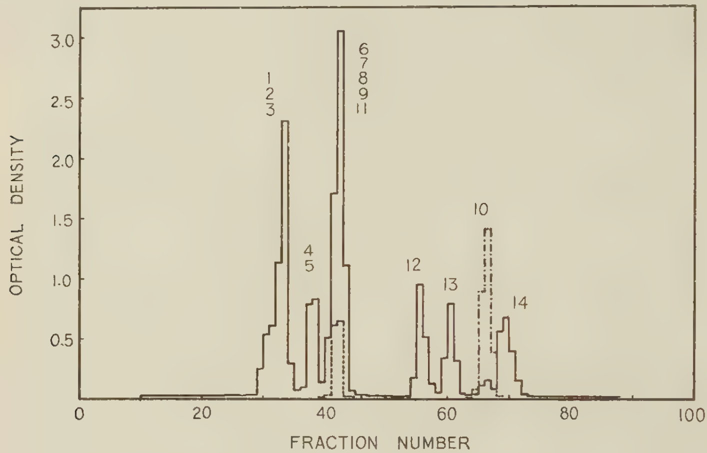

FIG. 1. Elution pattern of phenolic acids. Ultraviolet absorption was measured at 260 mμ (full lice), 295 mμ (dotted line ————————) and 330 mμ (dotted line ---------). Adsorbent: Amberlite IRC 50 (H^+-form). Eluent: solvent A. Column size: 0.76×95 cm. Room temperature: 30°. One fraction: 40 drops. Flow rate: 1.5 drops/min.

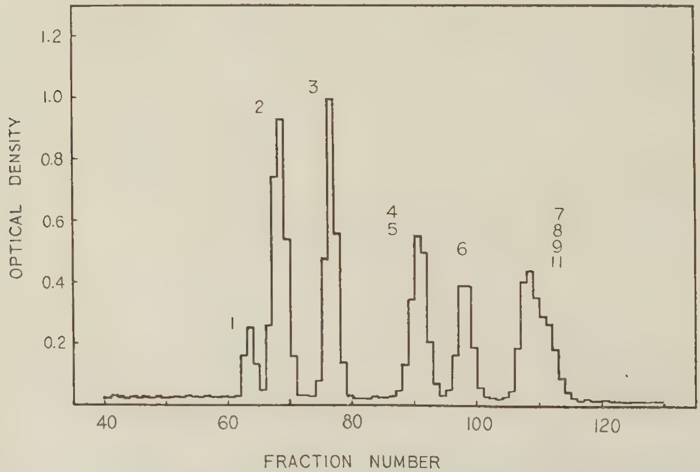

FIG. 2. Elution pattern of phenolic acids. Ultraviolet absorption was measured at 280 mμ. Adsorbent: Amberlite IRC 50 (H^+-form). Eluent: solvent B. Column size: 0.76×112 cm. Room temperature: 30°. One fraction: 20 drops. Flow rate 2 drops/min.

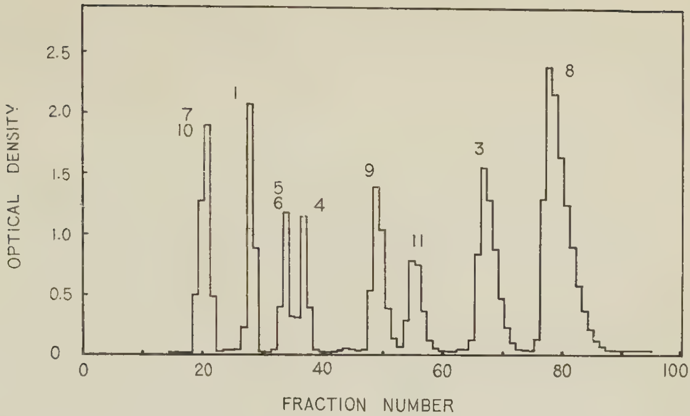

FIG. 3. Elution pattern of phenolic acids. Ultraviolet absorption was measured at 320 mμ (fraction 15 to 23), at 290 mμ (fraction 24 to 31), at 280 mμ (fraction 32 to 62) and at 260 mμ (fraction 63 to 95). Adsorbent: Duolite C 25 (Na^+-form). Eluent: solvent C. Column size: 0.8×95 cm. Temperature: 30°. One fraction: 40 drops. Flow rate: 1.5 drops/min. Homoprotocatechuic acid overlapped with *o*- and *m*-hydroxyphenylacetic acid.

SUMMARY

Chromatographic separation of phenolic acids was performed using Amberlite IRC 50 and Duolite C 25 as adsorbents. When Duolite C 25 was used in Na^+-form, citrate buffer (pH 4.34 ± 0.02, $M/10$) containing 20 per cent (v/v) of ethanol was used as the eluent. Mixtures of dilute hydrochloric acid, acetone and methylethylketone (9 : 1 : 2 and 6 : 1 : 2) were used for Amberlite IRC 50 in H^+-form. It was shown that this procedure successfully separated mono- and dihydroxylated benzoic and phenylacetic acid with a recovery of 80 to 90 per cent. Homogentisic acid gave a recovery of 65 per cent when it was separated on the column of Amberlite IRC 50.

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The Journal of Biochemistry, Vol. 46, No. 12, 1959

ON THE ACTION OF EGG WHITE LYSOZYME ON GLYCOL CHITIN

Lysozyme is one of the simplest enzymes and its chemical and physical characteristics as a protein have been elucidated considerably. However lysozyme is poorly characterized as an enzyme, since its activity has been mainly determined by lysis of bacteria such as *Micrococcus lysodeiktikus*. Therefore, studies on the relationship between the structure and the biological function of lysozyme have not yet been made. One of the authors has investigated the denaturation of lysozyme by heat, urea, and surface (*1*). However, changes in activity of lysozyme by denaturation could not be examined owing to lack of a suitable substrate.

In 1957, Berger and Weiser reported that lysozyme hydrolyzes chitin and has β-glucosaminidase activity (*2*). However the quantitative determination of lysozyme could not be made due to the complete insolubility of chitin in water and to the considerable slow rate of its hydrolysis. We have now found that lysozyme can actively catalyze the hydrolysis of glycol chitin which, in contrast to chitin itself, is readily soluble in water. Although it is as yet to be explored if this catalytic activity of lysozyme is actually involved in its bacteriolytic action, this finding seems to be of considerable importance in the study of the relationships between the structure and enzymatic functions of the lysozyme molecule. In this communication we wish to describe briefly some observations on the lysozyme-catalyzed hydrolysis of glycol chitin.

Lysozyme was prepared from hens' egg white by the direct crystallization method. It was recrystallized four times and finally lyophilized. Glycol chitin was kindly supplied by Dr. R. Senzyu, who prepared it by glycolation of chitin with ethylene oxide (*3*). The activity of lysozyme was determined by the rate of decrease in viscosity of glycol chitin solution after the addition of lysozyme. Without addition of lysozyme, the viscosity of the glycol chitin solution was unchanged by pH and temperature. The effect of pH on the change in viscosity of glycol chitin hydrolyzed by lysozyme is shown in Fig. 1. The maximal decrease in viscosity was attained at pH 3.5. The value for the optimum pH as determined by lysis of bacteria was markedly divergent, being between pH 5 and 7.4. The optimum temperature, as determined by using glycol chitin, is at 50° at pH 3.5. The optimum temperature, which was determined by lysis of bacteria, was between 55° and 60° (*4*). The rapid rate of decrease in viscosity of glycol chitin after addition of lysozyme shows that lysozyme splits glycol chitin molecules at random. The hydrolytic cleavage of the glycosidic linkages in glycol chitin was confirmed by the increase in the reducing power which took place

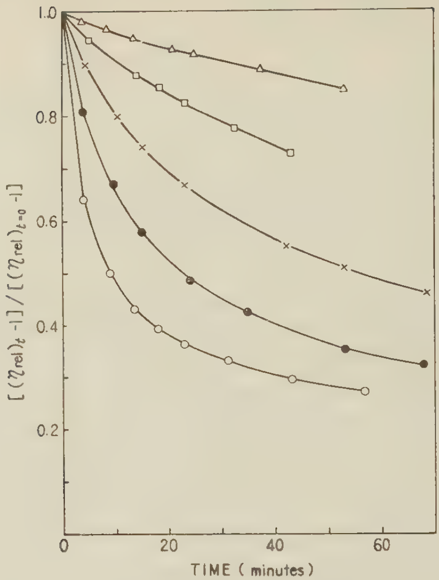

FIG. 1. The effect of pH on the rate of decrease in viscosity of glycol chitin hydrolyzed by lysozyme. Concentration of glycol chitin: 0.175 per cent. Concentration of lysozyme: 1.9 μM. △, pH 1.75; ○, pH 3.5; ●, pH 5.5; ×, pH 7.7; □, pH 8.5.

simultaneously with the viscosity decrease.

As described above, the activity of lysozyme can be readily determined by viscosity, using glycol chitin as a substrate.

Utilizing glycol chitin as a substrate, Ohdakara (*5*) found that the optimum of a liquifying chitinase obtained from *Aspergillus niger* measured by viscosity was pH 3.8 at an optimum temperature of 50°. It is very interesting that the enzymatic character of lysozyme resembles that of the chitinase.

The authors wish to express their hearty thanks to Dr. R. Senzyu of Kyushu University who supplied glycol chitin.

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PURIFICATION AND ASSAY METHOD OF PYRUVATE KINASE FROM BAKER'S YEAST

The existence of pyruvate kinase in yeast has been pointed out by many workers (*1–3*), but its purification has not been achieved for a long time. Therefore, we do not have enough evidence to elucidate whether the reaction mechanism of pyruvate kinase in yeast would be the same as that in muscle. This communication deals with the purification of the enzyme and the improvement of the assay of the enzyme activity.

Maceration juice of baker's yeast extracted with 0.06 M phosphate buffer of pH 7.2 at 37° for 3 hours was subjected to ammonium sulfate fractionation (0.40–0.60 saturation). The precipitate was further purified by a subsequent fractionation with ammonium sulfate. Initially the spectrophotometric method of Negelein (*4*), in which pyruvate kinase reaction is coupled with that of lactic dehydrogenase, was applied in the enzyme assay. The maximal activity of the crude preparation which was measured by this method was found in the fraction between 0.65 and 0.77 ammonium sulfate saturation, and the preparation was further purified by alumina C_{γ}-gel fractionation.

However, the preparation obtained here has shown the following properties when the enzymic activity was measured by the method of Negelein: 1) Lactate and pyruvate were not found as the reaction product either in the presence or absence of DPNH*, 2) the oxidation of DPNH was observed even in the absence of lactic dehydrogenase and KCl, 3) the oxidation of DPNH occurred more rapidly when ATP was replaced with ADP, 4) PEP was metabolized rapidly also without the addition of DPNH and ATP, and 5) the stoichiometric liberation (1 : 1) of orthophosphate was observed in the complete system with disappearance of PEP. Moreover, the presence of aldolase was proved by the method of Christian (*5*) and the formation of hexose could be detected by the Ashwell's method (*6*). These findings indicate the occurrence of a reaction sequence to reverse glycolysis instead of the expected pyruvate kinase reaction. In addition, another difficulty of using Negelein's method was the fact that lactic dehydrogenase contained a detectable activity of pyruvate kinase even in crystalline state. From the results above mentioned, it appears that the Negelein's method is not suitable for the assay of yeast pyruvate kinase.

Therefore, as an attempt for the enzyme assay, it was taken to determine the pyruvate formed in the reaction by the method of Friedemann and

* Following abbreviations were used: DPNH, reduced diphosphopyridinenucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PEP, phosphoenolpyruvate; DEAE-cellulose, diethylaminoethyl-cellulose; ATPase, adenosine triphosphatase.

Haugen (*7*), but in this case the selection of adequate pH of the reaction system was necessary because of the presence of alkaline phosphatase. When the reaction was carried out at pH 7.4, the fraction of 0.65–0.77 ammonium sulfate saturation was active in liberating pyruvate even without addition of ADP, in agreement with the liberation of inorganic phosphate. Therefore, the pH of the reaction system was selected at 5.8, where no phosphatase activity was detected and the pyruvate kinase activity could be determined exclusively.

With the use of this method for the enzyme assay, the maximal activity was found in the fraction of 0.45–0.55 ammonium sulfate saturation, and the fraction was further purified by the treatment with DEAE-cellulose-(Cl^-) after sufficient dialysis against 0.005 M phosphate buffer of pH 7.4. The fraction eluted from DEAE-cellulose between 0.005 M phosphate buffer of pH 7.4 and 0.025 M phosphate buffer of pH 6.0 showed about 15-fold increase in specific activity compared with the original extract (Table I). No or negligible activity of ATPase, adenylate kinase, phosphatase, and nucleoside diphosphokinase were detected in the final preparation.

TABLE I

Summary of Purification of Pyruvate Kinase from Baker's Yeast

Fractions	Total protein (*mg.*)	Total units	Specific activity (*units/mg.*) *of protein*)	Recovery (%)
1. Crude extract	1887	1510	0.8	100
2. First $(NH_4)_2SO_4$-fraction (0.40–0.60 saturation)	447	786	1.8	52
3. Second $(NH_4)_2SO_4$-fraction (0.45–0.55 saturation)	89	314	3.5	21
4. Supernatant of dialyzed enzyme	69	269	3.9	18
5. DEAE-cellulose fraction	12	165	14.1	11

A mixture (3.0 ml.) of 3×10^{-3} M PEP, 10^{-3} M ADP, 2.4×10^{-2} M $MgSO_4$, 2.25×10^{-1} M KCl, 3.3×10^{-2} M Tris-maleate buffer, pH 5.8 and 10 μg. of enzyme was incubated at 25° for 20 minutes. Immediately after incubation the mixture was submitted directly to the determination of pyruvate by the method of Friedemann and Haugen (*7*). One unit of enzyme was defined as the amount which causes the formation of 1 μ mole of pyruvate per minute under the condition specified as above.

Properties of the enzyme showed similarity to its counterpart in muscle. Concomitant formation of ATP and pyruvate was observed by paper chromatography with each two solvent systems. Stoichiometric relation between the formation of ATP and Pyruvate and the decrease of ADP and PEP was found to be in molar ratio each. Simultaneous presence of Mg^{++} or Mn^{++} and K^+ or NH_4^+ was essential for the enzymic activity in contrast to the observation of Muntz (*2*). *p*-Chloromercuribenzoate and heavy metal (Cu^{++}) completely

inhibited the enzymic activity at concentrations of $10^{-5}\,M$ or less. The optimum pH was found to be 5.8 when ADP was used as a phosphate acceptor. Nucleotide specificity of the enzyme at the reaction con- dition indicated in Table I was observed in the ratio, ADP : UDP : CDP : GDP : IDP= 100 : 10 : 6 : 5 : 3, which was somewhat different from the data of Strominger (*8*) and of our own (*9*) found in muscle enzyme.

These findings showed no substantial difference from those of muscle enzyme except the nucleotide specificity if at all.

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BACTERIAL FORMATION OF GLUTAMIC ACID FROM ACETIC ACID IN THE GROWING CULTURE MEDIUM

In the course of our preliminary studies on the glutamate formation from glucose, it was observed that a large amount of α-ketoglutaric acid or glutamate (in the presence of ammonium salts) was aerobically formed from acetate as well as from glucose by a cell suspension of *Brevibacterium flavum*, No. 2247, which had grown on glucose as the sole source of carbon and accumulated L-glutamate in its growing culture medium.

The experiments reported in the previous papers (*1*, *2*) provided evidence that acetyl-coenzyme A could react both with oxaloacetate to form citrate and with glyoxylate to form malate, and that glyoxylate necessary for this latter reaction could be provided by the aldol fission of isocitrate. Therefore, a modified tricarboxylic acid cycle with "the glyoxylate bypass" (*3*) was suggested as the possible pathway of glutamate formation from acetate in this bacterium (*1*).

Further experinents revealed that a considerable amount of glutamate (21.5 and 15.9 g./litre for medium A and B, respectively) was produced in the culture medium when this bacterium was grown on the following media, which contained acetate as the sole source of carbon:

Medium A—NH_4-acetate, 15 g. (as acetic acid); Na-acetate, 25 g. (as acetic acid); KH_2PO_4, 2 g.; $MgSO_4 \cdot 7\,H_2O$, 0.4 g.; Fe^{++}, 2 p.p.m.; Mn^{++}, 2 p.p.m.; thiamine hydrochloride, 0.1 mg.; corn-steep-liquor, 2 g.; and water to 1 litre (pH 8.0, 25 ml. per 500 ml. -flask).

Medium B—the contents were the same as medium A except for the addition of 0.3 μg. per litre of biotin instead of corn-steep-liquor.

Shaken at 30° for 71 hours. pH was checked occasionally and adjusted to 7.0–8.5 by the addition of acetic acid. The total amount of acetic acid used was 67 g. and 55 g. per litre for medium A. and B, respectively.

Brevibacterium lactofermentum No. 2256, *Brev. roseum* No. 7, and *Corynebacterium nov. sp.* No. 410 also excreted the following amounts of L-glutamate when medium A was used: 23.0, 21.5, and 6.9 g. per litre medium, respectively.

Glutamate production from acetate in these bacteria as well as formation of fumarate and citrate from acetate in molds (*4*, *5*) seems to provide a convenient tool for further studies on the metabolic pathway of two-carbon compounds.

None of the other bacteria tested thus far (*Pseudomonas ovalis* AHH-18, *Pseudomonas fluorescens* AHH-30, *Serratia marcescens* AN, *Proteus vulgalis* YO-10, *Aerobacter aerogenes* ATCC 8329, *E. coli* 151-9, *Bacillus megaterium* Y6, *B. subtilis* W-31, *B. circulans*, *B. pumilus* 134-a, *B. pumilus* 119-a, *Corynebacterium* No. 58, *Corynebacterium equi*, *Corynebacterium fasciens*, *Brevibacterium ammoniagenes* ATCC

6371 and 6372, *Brev. helvolum* ATCC 11822, *Brev. imperiale* ATCC 8365, *Brev. acetolyticum* ATCC 953 and 954, and *Micrococcus varians* ATCC 399) produced any detectable amount of glutamate.

Further details of the conditions of culture for the glutamate production will be published elsewhere.

The authors are indebted to Dr. H. Oeda and Mr. N. Motozaki of our laboratory for their interest and encouragement during the course of this work, and to the Institute of Applied Microbiology, University of Tokyo, and the Institute of Food Microbiology, Chiba University, for the gift of the bacterial strains.

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METHYLATION OF GENTISIC ACID

FORMATION OF 5-METHOXYSALICYLIC ACID

Recently Price (*1*), Axelrod (*2*), Armstrong (*3*), DeEds (*4*) and Kraychy (*5*) reported that the hydroxy-groups of xanthurenic acid, catechol derivatives, and sterols were methylated (*o*-methylation) to form their respective methoxyderivatives. It is known that gentisic acid, when administered to animal, is excreted in the urine as a glucuronate or etherial sulfate. This letter deals with the *in vivo o*-methylation of gentisic acid in man.

5-Methoxysalicylic acid was synthesized by the method of Graebe *et al.* (*6*) from gentisic acid.

100 mg. of gentisic acid were given orally to four healthy human subjects, and the urine was collected over a 12 hour period using HCl as a preservative. To hydrolyze the conjugated compounds, the urine was heated at 100° for an hour after addition of hydrochloric acid to a final concentration of 1 *N*. Under these conditions it was confirmed that no 5-methoxysalicylic acid was decomposed. The urine thus treated was passed through a 1.5×15 cm. column

TABLE I

R_f Values of Compounds on Paper Chromatogram

Solvent	5-Methoxy-salicylic acid	Gentisic acid	5-Methoxy-salicylic acid from urine
Isopropanol : ammonia (30%) : water (8 : 1 : 1)	0.65	0.57	0.65
n-Butanol : ammonia (30%) : water (15 : 1 : 4)	0.62	0.28	0.62
Benzene : acetic acid : water (2 : 2 : 1)	0.97	0.30	0.97
Other observations			
Ammoniacal $AgNO_3$	very weak	strong positive	very weak
UV-absorption (maximum)	318 mμ	320 mμ	318 mμ
Fraction No. of column chromatogram (0.8×100 cm. column; one fraction consists of 40 drops)	84–87	37–41	84–87

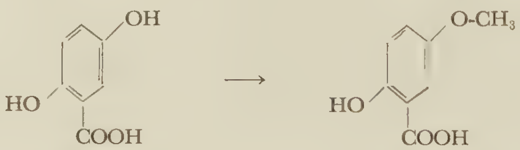
of Amberlite IRC 50 (H^+ form) 100–200 mesh. The column was then washed with 30 ml. of 0.1 *N* HCl, and the washing was extracted three times with

an equal volume of benzene. 5-Methoxysalicylic acid was readily extracted with benzene, but little gentisic acid was extractable by this solvent. The benzene layer was concentrated *in vacuo* to about 30 ml., and reextracted four times with 0.5 M phosphate buffer, pH 7.2. The combined extract was acidified with 25 per cent H_2SO_4 and extracted again three times with and equal volume of ether. The ether was evaporated and the residue dissolved in 0.2 ml. of distilled water was subjected to paper chromatography.

The R_f values of authentic 5-methoxysalicylic acid and gentisic acid are listed in the Table I. Paper chromatograms of the fraction from urine shows that 5-methoxysalicylic acid was excreted in the urine. 5-Methoxysalicylic acid was found in the urine of all four subjects who received gentisic acid, but was not found before gentisic acid administration.

The portion of the paper chromatogram corresponding to 5-methoxysalicylic acid was cut off, and extracted with distilled water. The extract was chromatographed on the column of Amberlite IRC 50 (H^+ form) and eluted with a solvent mixture: methyl ethyl ketone- acetone- 0.2 N HCl (2 : 1 : 6 v/v). Methoxysalicylic acid and gentisic acid appeared in separate fractions. The former compound coincided on the chromatogram with an authentic sample and also had the same absorption spectrum. The absorption spectrum of 5-methoxysalicylic acid differs from that of salicylic acid, which has an absorption maximum at 293 mμ.

Therefore, gentisic acid is thought to be methylated as follows:



Gentisic acid → 5-Methoxysalicylic acid

5-Methoxysalicylic acid has been identified previously in the scent glands of beavers (*7*). It is of interest to note that this compound arises from gentisic acid in human subjects.

Previously gentisic acid was detected in the urine of alcaptonurics (*8*). An attempt to isolate 5-methoxysalicylic acid and a study on the biological methylation of homogentisic acid are now in progress.

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THE CHEMICAL NATURE OF THE SO-CALLED 'TETRAHYDROXYNORSTEROCHOLANIC ACID' ISOLATED FROM THE GIGI-FISH BILE

The above acid was isolated originally by Ohta (*1*) from the Gigi-fish bile, and since then there appeared several reports on this acid found in some other sorts of fish bile (*2*) and even in avian bile (*3*, *4*).

A tentative formula, '3,6,12,24-tetrahydroxynorsterocholanic acid' suggested by Ohta (*1*) was largely confirmed by Isaka (*5*), the conclusive evidence that it is of a C_{27}-bile acid structure, however, being still lacking.

Recently Haslewood and his coworkers (*4*) isolated the very acid from the bile of king penguin and reported that elementary analyses of its ethyl ester "gave figures corresponding to $C_{26}H_{44}O_5$, *i.e.* to ethyl cholate, rather than to the formula $C_{29}H_{50}O_6$ required for ethyl ester of a 'tetrahydroxynorsterocholanic acid', $C_{27}H_{46}O_6$". This statement urged us to reexamine the chemical nature of this acid.

The Ohta acid was obtained from the hydrolyzed bile (Gigi-fish) according to the procedure described by Isaka *et al.* (*2*). Methyl ester of this acid was prepared by boiling it in menthanol containing 1 per cent of hydrochloric acid. Purification was carefully conducted by chromatography (Brockmann's alumina) and by repeated recrystallizations from ether-petroleum ether to give needles of m.p. 207° (Found: C, 70.19; H, 10.29. $C_{25}H_{42}O_5 \cdot C_4H_{10}O$ (etherate) requires C, 70.10; H, 10.56. Weight loss (120°, 5 hrs): Found 17.8 per cent; Calcd. 18.8 per cent)*. Elementary analyses of the solvent-free sample (m.p. 209°) gave the following figures: Found C, 71.27; H, 10.09. $C_{25}H_{42}O_5$ requires C, 71.09; H, 9.97 ($C_{28}H_{48}O_6$ requires C, 69.94; H, 10.07). This purest sample gave a positive Hammarsten reaction, just as previously reported (*1*).

Methyl ester of the acid (200 mg.) was oxidized with the chromic acid-sulfuric acid mixture according to Bladon *et al.* (*6*). The neutral oxidation product** was recrystallized from ethyl acetate, dilute alcohol and dilute acetone, successively, to give 120 mg. of needles decomposing at 210–224°. This product seems to be identical with methyl 'β-tetraketonorsterocholanate' of Ohta (m.p. 206–207°). The analytical figures of our sample are well corresponding to the formula $C_{25}H_{36}O_5$ (Found: C, 72.55; H, 8.89. Calcd.: C, 72.11; H, 8.65; $C_{28}H_{40}O_6$ requires C, 71.18; H, 8.47).

* The analytical data of the ester or the free acid in the previous papers might be of partially solvated samples (1, 3).

** The neutral oxidation product obtained by the usual CrO_3-acetic acid oxidation melted at 178–179°, just like methyl ester of 'α-triketocholanic acid' of Ohta (*1*).

The oxime of this product melted at 245° (m.p. of Ohta's sample: 239°) with decomposition (Found: C, 65.10: H, 9.04; N, 8.98. $C_{25}H_{39}O_5N_3$ requires C, 65.07; H, 8.46; N, 9.11).

The above oxidation product (200 mg.) was reduced by the modified Wolff-Kishner method (*7*), to give a mixture of cholanic and allocholanic acids, while the Clemmensen reduction of 'α-triketocholanic acid' was said to afford almost exclusively allocholanic acid (*1*). The reduction product was transformed into methyl ester (methanol-HCl), and subjected to chromatography (Brockmann's alumina). A fraction eluted by benzene-petroleum ether (1:9) was hydrolyzed and acidified. Crystals so obtained were recrystallized from dilute alcohol. m.p. 160-161° (Found: C, 79.99; H, 11.41. $C_{24}H_{40}O_2$ requires C, 79.93; H, 11.11).

A portion of this acid was converted to propyl ester as usual, recrystallized from dilute alcohol, and two different forms of crystals were obtained in about equal amounts. The one (fine needles) melted at 93.5–95.5° after several recrystallizations, showing no melting point depression (m.m.p. 97–104°) on admixture with authentic propyl allocholanate (m.p. 103 104°; free acid 171°). The other (prisms), obtained from the mother liquor of the above ester melted at 52–53° and the melting point was not elevated by further recrystallization from dilute methanol, being undepressed (m.m.p. 54.5–57°) on admixture with authentic propyl cholanate (m.p. 56.5–57.5°).

Another portion of the above reduction product (m.p. 160–161°) was methylated with methanol and conc. HCl. And the crystals obtained were likewise separated into 2 different forms under microscope; the one, needles, melting at 91.5–92.5° and the other, platelets, melting at 76.5–78.5°.

These esters showed no melting point depression on admixture with the corresponding esters of allocholanic and cholanic acids (m.p. 91–92°; m.p. 87.5–88.5°, respectively).

From these data it is clearly indicated that (i) 'tetrahydroxynorsterocholanic acid' is not a bile acid of the C_{27} type with four OH groups, but an isomer of cholic acid, exactly as has been suggested by Haslewood *et al.* (*4*), and that (ii) the asymmetric center of C_5 was involved when its oxidation product was reduced by the Wolff-Kishner method as well as by the Clemmensen (*1*). The latter conclusion was well substantiated by the experimental data of Isaka (*5*), who had been able to transform the Ohta acid into hyodeoxycholic acid. If the presence of C_{12}-OH group in the Ohta acid molecule was correct as was indicated by Isaka (*5*), this acid would be of the structure, $3\alpha,6\alpha,12$-trihydroxycholanic acid. This conclusion was strongly supported by the recent finding (*8*) that a new metabolite of deoxycholic acid, $3\alpha,6\beta,12\alpha$-trihydroxycholanic acid (m.p. 135-137°) gave on the usual CrO_3-acetic acid oxidation 2 different kinds of triketocholanic acid (m.p. 198° and m.p. 234°, respectively) exactly as the Ohta acid did. The single derivation of the keto acid of higher melting point (as methylester, probably of 3,6,12-triketoallocholanic acid) in the present experiment can be interpreted as follows: Since the CrO_3-oxidation of the Ohta acid (as methyl

ester) was here conducted in the presence of sulfuric acid (according to Bladon *et al.* (*6*)), the 6-ketocholanic acid derivative then formed was smoothly and totally converted into the allo-series.

Since $3\alpha,6\alpha,12\alpha$-trihydroxycholanic acid prepared synthetically (*9*) was found to be quite different from the Ohta acid, the probable structure of this acid would be $3\alpha,6\alpha,12\beta$-trihydroxycholanic acid* and a reexamination with another sample of this acid will be carried out later on.

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* A possible formula $3\alpha,6\beta,12\beta$-trihydroxycholanic acid, might be here reserved.

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AN EXCHANGE OF β-HYDROGEN OF AMINO ACID WITH MEDIUM WATER BY TRANSAMINASE ACTION

It is known that the hydrogen atom attached to α-carbon of an amino acid exchanges with hydrogen atom of medium water during the course of the transaminase action (*1*, *2*). The authors found that the hydrogen atoms on β-carbon exchanged as well by the enzyme action.

42 mg. of L-alanine, 20 mg. of transaminase preparation (purified upto the stage of the third fractionation by ammonium sulphate, according to Green and others' method (*3*), dialysed and lyophilized), each trace amount of α-ketoglutaric acid and pyridoxal phosphate were dissolved in 5 ml. of 0.08 M phosphate buffer (pH 7.0) in 99.8 per cent deuterium oxide. The final pH was adjusted to 7.0 with concentrated sodium hydroxide in D_2O. One drop of tolune was added as anticeptic. After 38 hours' incubation at 38°, the reaction mixture was boiled and filtered. The amino acid in the filtrate was absorbed on a column (1.8 cm.$^2\times$7 cm.) of Amberlite IR 112, H^+ type. The column was washed with distilled water and the amino acid was eluted with 0.15 N ammonium hydroxide and dried under vacuum. The alanine recovered was dissolved in 10 ml. of water and passed through another column of Amberlite IR 45, acetate type, to remove acidic impurities. The solution was evaporated to dryness under vacuum. The alanine, obtained as the evaporation residue, was dissolved again in a minimum amount of water and recrystallized by the addition of ethanol, and dried under vacuum at 100°. 33 mg. of L-alanine were recovered.

The alanine preparation obtained here contained 3.76 atoms of deuterium per molecule, according to the mass spectrometric analysis (*4*). It is evident that these four deuterium atoms are attached to α- and β-carbons of the alanine, because other three hydrogen atoms in the molecule are readily exchangeable with medium water and replaced by normal hydrogen during the course of isolation.

Fig. 1 shows the infra red absorption spectrum of this α,β-tetradeuterio-L-alanine. The spectra of normal L-alanine and chemically synthesized α-deuterio-DL-alanine are also shown for comparison. The latter was synthesized by the electrolytic reduction of α-isonitrosopropionic acid in deuterium oxide. The pattern of the absorption spectrum clearly shows the disappearance of the peaks of α-CH (deformation, 1308 cm.$^{-1}$) and $-CH_3$ (degenerating deformation, 1451 cm.$^{-1}$ and symmetric deformation, 1356 cm.$^{-1}$) groups (*5*).

10 mg. of the α,β-tetradeuterio-L-alanine was treated again with transaminase in the presence of pyridoxal phosphate and α-ketoglutaric acid in 4

ml. phosphate buffer in ordinary water. 7 mg. of alanine were recovered from the reaction mixture. The infra red spectrum of the re-treated alanine agreed completely with that of normal L-alanine.

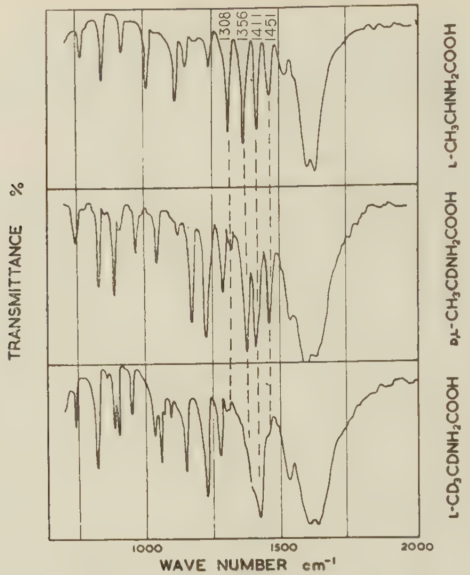

FIG. 1. Infra red absorption spectra of L-alanine, α-deuterio-DL-alanine and enzyme treated L-alanine.

TABLE I

The Incorporation of Deuterium into α and β Positions

Alanine Preparation	Percent decrease in	
	α-CH	$-CH_3$
10 minutes	13	14
25 minutes	20	29
16 hours, with boiled enzyme	2	0
16 hours, without enzyme	0	0
Chemically synthesized α-deuterio-D, L-alanine	>90	7

The rates of exchange at positions α and β were compared in D_2O by following the rates of decrease in the heights of infra red absorption peaks at 1451 cm.$^{-1}$ and 1308 cm^{-1}. The absorption at 1411 cm.$^{-1}$, which remained unchanged and was assigned to $-COO^-$ symmetric stretching, was taken as standard. The results are summarized in Table I. The values obtained with chemically synthesized α-deuterio-DL-alanine and with L-alanine recovered from the reaction mixture of control experiment with boiled enzyme or

without the enzyme, are also given in the Table. It can be seen the rate of exchange is greater at β position than at α position.

From these results, the authors propose a reaction mechanism for transaminase in which the dissociation of a proton from β-carbon is an essential and primary step of the activation of the Schiff's base which is formed from the amino acid and pyridoxal phosphate. The dissociation at β position seems to be more conceivable than that at α position (*6–8*), according to the present knowledge of electronic theory, and can explain a number of facts known for enzyme reactions, which require pyridoxal phosphate as the cofactor.

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